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Psoroptes ovis mange in Belgian Blue cattle:

How to approach a multifactorial problem?

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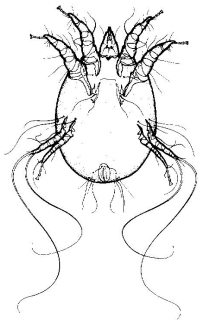
List of abbreviations

ABC	ATP binding cassette
AP	activator protein
APC	antigen presenting cell
AREG	amphiregulin
ATP	adenosinetriphosphate
BB	Belgian Blue
BW	body weight
CCL	chemokine C-C motif ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CI	clinical index
ConA	concanavalin A
COX	cyclooxygenase
Cu	copper
CXCL	chemokine C-X-C ligand
DC	dendritic cell
DPBS	Dulbecco's phosphate buffered saline
EDC	epidermal differentiation complex
ELISA	enzyme-linked immunosorbent assay
ES	excretion/secretion
EST	expressed sequence tag
ETS	protein C-ets
FACS	fluorescence-activated cell sorting
FCERA	Fc IgE receptor alpha
Fe	iron
FIL	filaggrin
Foxp3	forkhead box P3
FV	farm visit
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte-colony-stimulating factor
GF-AAS	graphite furnace atomic absorption spectrophotometry
GluCL	glutamate-gated chloride channel

HDAC	histone deacetylase
HE	haematoxylin-eosin
HF	Holstein Friesian
HPRT	hypoxanthine phosphoribosyltransferase
ICAM	intercellular adhesion molecule
IDT	intradermal skin test
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
Ig	immunoglobulin
IVL	involucrin
kDa	kilodalton
kg	kilogram
KOH	potassium hydroxide
LA	long acting
LOR	loricrin
LPS	lipopolysaccharide
MC	mite count
mg	milligram
µCi	microcurie
µg	microgram
ML	macrocyclic lactone
ml	millilitre
µl	microlitre
mm	milimeter
µm	micrometer
MYD	myeloid differentiation
NCBI	National Centre for Biotechnology Information
NCR	natural cytotoxicity triggering receptor
ND	not determined
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NK	natural killer
OOS	opisthosomal setae
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell

PCA	passive cutaneous anaphylaxis
PCR	polymerase chain reaction
PI	proliferation index
pi	post infection
PKH	Paul Karl Horan
PRR	pattern recognition receptor
Q	questionnaire
qRT-PCR	quantitative real-time polymerase chain reaction
RLP	ribosomal protein P
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RPS	ribosomal protein S
ScI	scratching index
SDHA	succinate dehydrogenase complex subunit A
Se	selenium
SEM	standard error of the mean
SI	stimulation index
SMIPP	scabies mite inactivated (serine) protease paralog
SMS	scabies mite serpin
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
SPRR	small proline rich proteins
TCR	T-cell receptor
TGF	transforming growth factor
Th	helper T-cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cell
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Zn	zinc



CHAPTER 1

Review on *Psoroptes ovis*

1.1 Introduction

Cattle can get infested with several mite species that occur worldwide: *Demodex bovis, Sarcoptes scabiei, Chorioptes bovis* and *Psoroptes ovis* (Taylor *et al.*, 2007). *Demodex bovis* is located in the hair follicles and sebaceous glands and although the mite can live as a commensal on the skin, it can also cause small cutaneous nodules, causing hide damage (Taylor *et al.*, 2007). Infections with *Sarcoptes scabiei (S. scabiei),* a mite that burrows into the epidermis, cause hair loss and thickened skin but have become rare in cattle in Belgium. *Chorioptes bovis (C. bovis)* mange is a relatively benign condition that is often located at the legs, tail base and udder and that mainly affects dairy cattle, such as Holstein Friesians (HF) (Mitchell *et al.*, 2012; Taylor *et al.*, 2007). *Psoroptes ovis (P. ovis)* infestations are the most aggressive mite infections, leading to severe pruritus, crusts, excoriation and secondary bacterial infections. The disease is highly contagious, affects mostly beef cattle breeds and causes important economic losses mainly due to weight loss and impaired leather quality (Cole and Guillot, 1987; Fisher and Wright, 1981; Pouplard and Detry, 1981; Rehbein *et al.*, 2003; Taylor *et al.*, 2007). The Belgian Blue (BB) beef breed seems to be highly susceptible to this infection and often has more severe and sometimes even generalized lesions (Bates, 1998; Losson *et al.*, 1999; Pouplard *et al.*, 1990). Although this mite species is ubiquitous in sheep, in cattle the geographical spread is more restricted (Losson *et al.*, 1999), with a focus in Belgium where the disease is endemic (Minihan *et al.*, 2002). Outbreaks of psoroptic mange in countries where *P. ovis* was previously eradicated often occur after importation of BB cattle (Jones *et al.*, 2014; Millar *et al.*, 2011; Minihan *et al.*, 2002; Mitchell *et al.*, 2012).

The classification of *Psoroptes* spp. mites has long been a subject of debate. The earliest information dates from 1838, when Hering determined 9 species based on the infested host (Pegler *et al.*, 2005). In 1861 Furstenberg hypothesized that all *Psoroptes*-mites belonged to the same species, *P. communis*, but that different varieties existed according to the host (Bates, 1999). Megnin and Raillet changed the species name in 1877 and 1893 respectively and in 1922 Hirst split the genus into *P. communis* and *P. natalensis* (Bates, 1999). From 1958 onwards the Sweatman taxonomy has been used, in which 5 species are differentiated: *P. cuniculi*, *P. cervinus*, *P. equi*, *P. ovis* and *P. natalensis*. This classification is based on the

morphology of the outer opisthosomal setae (OOS) of male mites and the location on and species of the host (Bates, 1999; Pegler *et al.*, 2005; Sanders *et al.*, 2000). However, recent research demonstrated a large inter- and intraspecies variation in the OOS length, making this parameter unreliable for species differentiation. Moreover, *P. ovis* and *P. cuniculi* crossbreds were able to produce viable offspring, genetic studies revealed little to no difference between mite species and complete antigenic cross-reactivity was demonstrated between mites from different hosts. Hence, it was concluded that these supposedly separated species are in fact one species (Sanders *et al.*, 2000). In conclusion, the classification of *P. ovis* can be based on several differentiation techniques, of which the practicality is still under discussion. In the present thesis the Sweatmann taxonomy is followed and *P. ovis* is used to indicate mites of cattle and sheep. These mites are suspected to be host adapted strains of the singular species *P. ovis* (Pegler *et al.*, 2005).

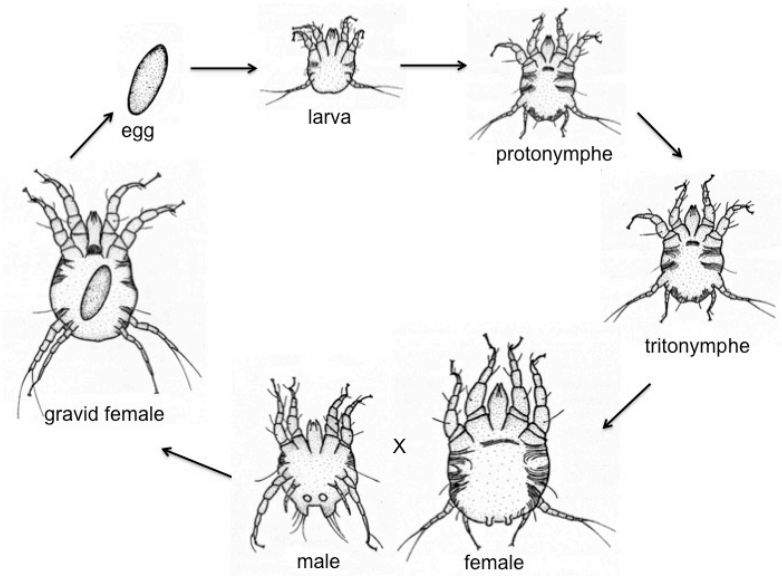
Causes of the increased susceptibility and the high prevalence of *P. ovis* infestations in BB cattle remain unclear (Bates, 1998; Losson *et al.*, 1999; Pouplard *et al.*, 1990), but as psoroptic mange is a multifactorial disease, environmental, epidemiologic and host immune parameters may influence the outcome of the disease on farm level and may partially explain the susceptibility of the BB breed. Therefore, in this introductory chapter, the available literature on *P. ovis* will be reviewed with the focus on life cycle, pathogenesis, epidemiology, diagnosis, treatment and the host immune reactions during infection.

1.2 Life cycle and epidemiology

The life cycle of *P. ovis* is direct and this obligate ectoparasite spends its entire life on the host (Figure 1). Gravid females produce large 250 µm long, oval eggs (Taylor *et al.*, 2007). A hexapod larva hatches from this egg and undergoes 3 moulting stages: a protonymph develops first, which later moults into a tritonymph. During these moulting phases, the mites do not feed and are immobile. Nymphs already have 4 pairs of legs and sexual dimorphism can be noticed from these stages onwards. The tritonymph finally moults into an adult *P. ovis,* which has several characteristic features: the body is oval, the mouthparts (chelicerae) are pointed and 3 out of 4 pairs of legs support 3 segmented pretarsi (pediculi) ending in funnel-like

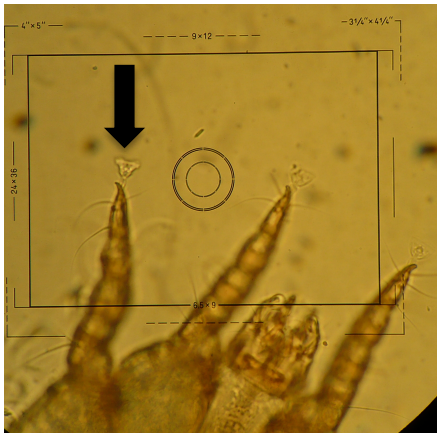
suckers, the pulvilli (Figure 10) (Taylor *et al.*, 2007). These mites do not have external tracheal openings (stigmata), which means gas exchange occurs directly through the cuticle (Bates, 2012). Adult males are smaller than females, have copulatory suckers and the fourth pair of legs is very short (Sanders *et al.*, 2000; Taylor *et al.*, 2007). They will mate with female tritonymphs and detach after the female has moulted into an adult. Insemination takes place just before the male detaches. After this, the female starts to produce about 2 to 3 eggs per day depending on the environmental temperature, and as the life span of a female is around 16 to 42 days, she will have laid 40 to 90 eggs in total (Bates, 1998 and 2012; Pouplard *et al.*, 1990; Taylor *et al.*, 2007; van den Broek and Huntley, 2003). The transition from one stage to another takes approximately 2 days (12 to 36 hours), which means the total life cycle from egg to adult takes about 10 up to 19 days (Bates, 2012; Gibbs *et al.*, 1986; van den Broek and Huntley, 2003; Wall *et al.*, 1999). Because of the large numbers of eggs produced by the females and the relatively short life cycle duration, the mite population will increase exponentially and double every 6 days (Wall *et al.*, 1999). The mites feed superficially by scraping the epidermis with their chelicerae, which will release proteins and serous exudate. By using a pre-oral trough, the mites can transport these nutrients to their pump-like pharynx. Sheep derived mites appear to feed mainly on lipid emulsions, which consist of skin cells and bacteria, but cattle derived mites also feed on blood serum and red blood cells (Bates, 1998, 2012; Kirkwood, 1985; Wright and Deloach, 1981). The digestion of nutrients in the mite gut mainly occurs through secretion of enzymes, such as cysteine proteinases, serine proteinases and metalloproteinases. In addition, luminal bacteria including *Serratia marcescens* and *Comamonas* spp. may be a direct or indirect food source, have an additional symbiotic digestive function and/or may be crucial for mite survival (Hamilton *et al.*, 2003).

Figure 1. Life cycle of *Psoroptes ovis.*



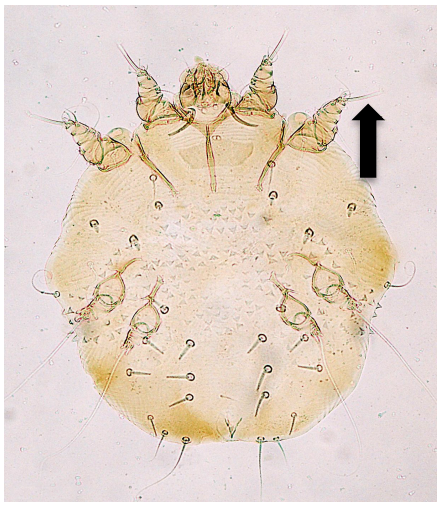
The life cycle of *Chorioptes bovis* is identical to that of *P. ovis* but the duration is about 3 weeks. Females only lay 1 egg per day and live for 2 to 3 weeks. The mites are smaller than *P. ovis* and have short, unsegmented pediculi ending in a large cup-shaped pulvillus (Figure 2). With their rounded mouthparts, the mites mainly feed on cell debris. They are located at the hind legs, tail base, groin and udder and cause less severe symptoms compared to *P. ovis* with formation of small, dry scales or crusts (Bates, 2012; Taylor *et al.*, 2007).

Figure 2. *Chorioptes bovis* with typical short pediculus and large pulvillus (black arrow) (x400).



Another obligate ectoparasite is *Sarcoptes scabiei*, the burrowing mite. Females live for 2 months and burrow deep in the skin to deposit their eggs (1 to 3 per day). In 3 to 4 days, the eggs hatch and the larvae either develop in the burrow, in hair follicles or on the skin surface. The larva moults to a protonymph, which develops into a tritonymph and finally an adult, with each moulting stage taking about 2 days. Adults mate on the skin after which the female tries to find a suitable place to make her permanent burrow. The whole cycle takes between 2 to 3 weeks. The mites have a round body and short legs, the pediculi are long and unsegmented and the pulvillus is small (Figure 3). The mites prefer less well haired areas and are often found at the tail base, the neck and the head. They cause severe pruritus and alopecia, lichenification and crust formation may occur (Bates, 2012; Taylor *et al.*, 2007).

Figure 3. *Sarcoptes scabiei* with typical long unsegmented pediculus (black arrow) (x250).



Mange is a winter disease, although in severe cases it can be seen all year round (Bates, 1998). Once introduced in a herd or farm, this highly contagious infection will rapidly spread through direct and indirect transmission. Direct or close contact between animals is the main transmission route of *P. ovis* infections (Mitchell *et al.*, 2012) and this statement indicates the importance of subclinical carriers. Carriers are clinically healthy but infested, although the mites seem to be in hibernation (Jones *et al.*, 2014; Mitchell *et al.*, 2012; Pouplard *et al.*, 1990). In sheep, 'latent phase' mites have been found around the ears, the perineum, the infra-orbital and interdigital fossae and the inguinal pouches (Bates, 2012; Kirkwood, 1985) and in

cattle at the base of the horns and the top of the head (Bussiéras, 1987). Introducing untreated, seemingly healthy carriers into a herd is therefore a major risk factor for introduction of the disease, as these animals can transmit the infection to uninfested herd members (Carty and Nisbet, 2011; Millar *et al.*, 2011; Mitchell *et al.*, 2012; Phythian *et al.*, 2013; Pouplard *et al.*, 1990). Even though *P. ovis* mites remain on the host throughout their entire life cycle, they are capable of surviving in the environment. Indirect transmission through contaminated housing infrastructure, bedding, tools, work wear and transportation machinery should therefore not be underestimated (Bates, 1998). Several studies on off-host survival time for *P. ovis* have been conducted and the longest period for sheep derived mites was 48 days (Smith *et al.*, 1999). As mites do not feed during this period, their viability will decline and consequently, their infectivity (capability of inducing an active infection in a new host) is about 2 weeks, depending on the environmental conditions. Low temperatures, high humidity and low exposure to direct sunlight are ideal for *P. ovis* survival and infectivity off the host (Bates, 1998 and 2012; van den Broek and Huntley, 2003). The influence of climatic conditions on the off host survival time of mange mites indicates that climate change could potentially have an effect on the disease. However, as an increasing global temperature will decrease off host survival and duration of the housing period, during which the highest transmission of mites occurs, climate change will most likely have a negative effect on mange mite abundance and spread. However, more research on the complex interactions between climate change and parasite population dynamics is necessary (Morgan and Wall, 2009).

For psoroptic mange, there is no specific predisposition for animals of a certain age or sex: the disease occurs as often in females as in males and all ages can get infested (Bates, 2012; Mitchell *et al.*, 2012; O'Brien, 1999). It should be stressed however that there is a clear breed susceptibility: while dairy cattle seem to be resistant and rather affected by chorioptic mange, beef breeds tend to frequently get infested with *P. ovis* and suffer from more severe lesions (Bates, 1998; Losson *et al.*, 1999; Pouplard *et al.*, 1990). Similarly, large differences in the progress and extent of *P. ovis* infections between sheep breeds have been documented, with fine wooled breeds presenting more severe and rapidly spreading lesions with high numbers of adult mites, compared to coarse wooled breeds (Bates, 2012; Fourie *et al.*, 2002; Smith *et al.*, 2001). In cattle, the possibility to self-groom, associated with the

presence or absence of large horns, differences in skin thickness and/or conformation and behavioural varieties have been suggested as reasons for breed susceptibility differences to mange (Lonneux and Losson, 1996; Lonneux *et al.*, 1998b and c). Based on experimental work on ticks, there are some additional hypotheses to explain why BB are more susceptible than other breeds, such as HF. Cattle breeds that are rather resistant towards tick infestations appear to have a smaller range of antigen recognition, which decreases the cellular inflammatory response, dampens the constant trigger of immune cell development and contains the advancement towards chronic inflammation (Piper *et al.*, 2010). This coincides with earlier work done on psoroptic mange, in which the suggestion is made that HF display a faster decline in *P. ovis* antigen reactivity, leading to a less intense immune response and less severe clinical signs (Losson *et al.*, 1999). Tick-resistant cattle breeds also appear to show a larger up-regulation of proteins that support collagen fibres, leading to a stronger skin barrier against tick infestation and potentially mite infections (Wang *et al.*, 2007). Physiological differences in general, especially in skin structure and composition could influence the easiness by which mites can breach the skin. Lastly, breeds that have been exposed to ticks for hundreds of years have co-evolved into responding less to the biologically active compounds of tick-saliva and as such, diminish the ability of the tick to modulate the skin in order to facilitate blood feeding (Piper *et al.*, 2010). Because the BB breed is a relatively 'new' cattle breed (Huyghe *et al.*, 2014), *P. ovis* might induce a much stronger cellular reaction compared to other cattle breeds and result in the severe clinical signs typical for these animals. Even within one breed, differences in susceptibility on group or individual level can be observed. Problem farms where all animals suffer from persistent and severe mange have been described for sheep (Phythian *et al.*, 2013) and have been suggested to exist in cattle practice. Environmental influences, the presence of resistant mite strains, concurrent infectious diseases with a potential immunosuppressive effect (e.g. bovine viral diarrhoea, infectious bovine rhinotracheitis, bovine malignant catarrhal fever…) or distinctive management strategies, such as specific feed composition, treatment (of mange and other diseases) and hygiene protocols, could explain the different clinical outcome on farm level (Minihan *et al.*, 2002; Phythian *et al.*, 2013; Wall, 2012). In sheep, differences in susceptibility between individual animals might be linked to the ability of the host to mount an allergic response to the mite, as some immunologically unresponsive individuals seem to develop little to no clinical signs. Furthermore, a

divergent individual bacterial skin flora may also be responsible for more 'resistant' individuals in the field. Some bacteria can have inhibitory effects on parasites and mites have to adjust to the bacterial flora of a new host before they can start feeding and reproducing. Individuals carrying an 'inhibitory' bacterial flora might be more resistant to infestation (Bates, 2012). Whether these are also the causes of varying individual sensitivity in cattle remains uncertain, but the presence of separate *P. ovis* strains with unusual virulence could be partly responsible. Although not yet described in cattle, research on sheep in the US demonstrated the presence of several distinct, seemingly more pathogenic, *P. ovis* populations that spread faster, caused more severe lesions, survived longer on the host and had a higher survival rate after treatment (Bates, 1997; Roberts and Meleney, 1970). Finally, within the sensitive BB breed, the presence of highly susceptible genetic lines could also explain the variety in clinical signs within an affected herd (Jones *et al.*, 2014; Minihan *et al.*, 2002; Pruett *et al.*, 1986; Stromberg and Fisher, 1986).

1.3 Pathogenesis and clinical signs

Psoroptic mange is a multifactorial disease: besides the presence of mites, nutrition, hygienic conditions, concomitant diseases, housing, climate, ventilation and other management factors, such as treatment protocol, may influence the presence or absence of a clinical mange infection (Mitchell *et al.*, 2012; O'Brien, 1999; Pouplard *et al.*, 1990). Under optimal conditions, the development of symptoms begins when the mites start feeding. *Psoroptes ovis* is a non-burrowing ectoparasite that lives superficially on the skin surface and by using its mouthparts, *P. ovis* actively scrapes and abrades the epidermis instead of piercing it (Bates, 2012; van den Broek and Huntley, 2003). This mechanical skin damage will cause leakage of proteins and serous exudate, which is one of the main nutrition sources for the mite (Bates, 1998; Kirkwood, 1985; Wright and Deloach, 1981). While the mites feed and reproduce, they release allergenic excretion and secretion (ES) products, such as faecal pellets, digestive fluids, enzymes and shed cuticle. As *P. ovis* mites damage the epidermis during feeding, the diffusion of these pro-inflammatory allergens into the skin is facilitated and they are thought to be the main initiators of clinical signs as they induce a hypersensitivity or allergic reaction in the host (Bates, 2012; Smith *et al.*, 2001; Stromberg and Fisher, 1986; van den Broek and Huntley, 2003; van den Broek

et al., 2004). When the mite population increases and the lesions spread, the released exudate dries out in the centre and forms a dry yellow crust or scab (Stromberg and Fisher, 1986; Taylor *et al.*, 2007), while at the edges the infection remains 'active' indicated by moist, inflamed skin (van den Broek and Huntley, 2003). In sheep, reproducing mites are usually found at the edge of these crusts, while in cattle they are thought to reside on the entire lesion (Bates, 1998; Losson *et al.*, 1999; Taylor *et al.*, 2007; van den Broek and Huntley, 2003).

Clinically, erythematous skin and multiple thick yellowish crusts can be seen (Figure 4), but in some cases milder clinical signs with more dandruff-like flakes are visible (Taylor *et al.*, 2007; van den Broek and Huntley, 2003). While erythematous skin can be noticed from one hour post-infection (pi) onwards in sheep, in cattle it takes about 7 days for clinical signs to appear (Stoeckli *et al.*, 2013; Stromberg and Fisher, 1986). Lesions are mainly seen at the tail base, back and withers, but the infestation spreads rapidly and can cover the entire body in severe cases (Bates, 1998; Millar *et al.*, 2011; Pouplard *et al.*, 1990; Stromberg and Guillot, 1987). Secondary bacterial infections often occur, causing abscesses or pyodermatitis. Moreover, the susceptibility to other infectious diseases, such as pulmonary infections may be increased (Bates, 1998; Stromberg and Guillot, 1987; Taylor *et al.*, 2007; van den Broek and Huntley, 2003). Most importantly, the allergic reaction and the irritation from the mites induce an intense pruritus, leading to self-traumatising behaviour, such as licking, scratching and biting. Although affected hosts will get rid of some mites this way, mechanical skin abrasion is the most important consequence leading to hair loss, skin damage, seromas, hematomas and bleeding wounds (Bates, 1998; Pouplard *et al.*, 1990; van den Broek *et al.*, 2000). All these factors will intensify the local intradermal inflammation, which leads to increased serum extravasation, creating the perfect microclimate for mites to survive. This ideal environment will in turn translate in an exponential growth of the mite population and the clinical lesions (Lonneux and Losson, 1996; Stromberg and Fisher, 1986; van den Broek and Huntley, 2003).

Figure 4. Dorsal view of a BB cow infested with *P. ovis* (left) and close-up of the clinical lesions (right). Typical clinical signs are alopecia, erythematous skin and yellow wet crusts.

Histopathologically, epithelial hyperkeratosis, dermal oedema, sebaceous gland hypertrophy, increased dermal thickness and marked perivascular dermatitis are observed with fast infiltration of eosinophils, neutrophils and lymphocytes to the site of infection. Mast cell hyperplasia and degranulation are also obvious, with further attraction and activation of eosinophils and the release of vasoactive substances as consequences. The latter will increase dermal oedema and vascular permeability, which in return will intensify the extravasation of serous exudate. Occasionally, epithelial microabscesses, pustules and ulcerations are noticed (Minihan *et al.*, 2002; Stromberg and Fisher, 1986; Stromberg and Guillot, 1989; Taylor *et al.*, 2007; van den Broek *et al.*, 2000 and 2004).

Systemic changes can be anaemia, neutropenia, lymphopenia, eosinophilia and increased plasma protein concentrations (Losson *et al.*, 1988; Stromberg *et al.*, 1986; Stromberg and Guillot, 1989; van den Broek *et al.*, 2000; van den Broek and Huntley, 2003).

In parallel with the expansion of the mite population, the host develops the *P. ovis* specific immunoglobulins (Ig) IgE, IgM and IgG, which negatively affect mite survival as they are excreted in the serous exudate and ingested by the mites (Bates, 2012; Pruett *et al.*, 1986; van den Broek *et al.*, 2000 and 2003b). On the other hand,

IgE antibodies might be partly responsible for the cutaneous pathology as they induce an IgE-mediated type 1 hypersensitivity reaction (van den Broek *et al.*, 2000). None the less, if this humoral response is sufficient, combined with the lack of new feeding sites for the mites, the further development of the mite population will stabilize, decrease and eventually be eliminated (Stromberg and Fisher, 1986). Compared to the response during primo-infection, the rapid increase in anti-*P. ovis* antibodies, especially IgE, during consecutive mange infections or after vaccination can even lead to a marked clinical resistance in sheep (Bates, 2000; Smith *et al.*, 2001 and 2002; van den Broek *et al.*, 2000).

Several distinct phases in the longitudinal progression of the disease have indeed been identified in sheep. At first, a subclinical or lag phase is observed, during which the mites adjust to the new host and low mite numbers and very small, almost undetectable lesions are present. Under field conditions, the lag phase might take up to 240 days before the rapid growth phase sets in. This phase, during which mite numbers, lesions and mite specific antibodies increase dramatically, is followed by a peak or plateau phase. The increase in mite numbers and lesion development slows down and eventually the decline phase is initiated. At this point, feeding sites for the mites have become scarce and the immune response of the host takes over, which is translated in a decreased mite population and less active clinical signs. Eventually, the regression phase begins, during which the clinical signs heal and all mites are eliminated (Bates, 2012). Natural recovery from the infection is therefore not unusual, but will depend on the host's previous exposure to *P. ovis*, the virulence of the mites and the host species and breed (Bates, 2012). An example of the latter is that compared to BB animals, self-cure in HF cattle is much more frequent (Bates, 1998). On the other hand, infested animals often get into the cryptic or 'carrier' stage of mange, in which they are clinically healthy but carry hibernating mites that can re-introduce the disease later on (Bates, 2012; van den Broek and Huntley, 2003). Finally, patients may also develop chronic mange, meaning they are unable to control the disease, which often leads to high mite numbers, dry crusts or flakes with excoriation, lichenification and hyperkeratinisation of the skin (Bates, 1998; Pruett *et al.*, 1986; Taylor *et al.*, 2007).

Psoroptic mange is a threat to the beef cattle industry as it causes animal welfare issues and important economic losses (Minihan *et al.*, 2002; Pouplard and Detry,

1981; Pouplard *et al.*, 1990). Skin damage induced by increased self-grooming behaviour will negatively influence hide quality (Figure 5) and in dairy cattle milk production might be impaired. Moreover, affected animals are restless and pruritic and therefore preoccupied with scratching and licking and distracted from feeding. Together with substantial protein loss through the damaged epithelial barrier, the lower feed intake may translate in weight loss or impaired weight gain in growing animals (Cole and Guillot, 1987; Fisher and Wright, 1981; Pouplard and Detry, 1981; Rehbein *et al.*, 2003; Taylor *et al.*, 2007; van den Broek and Huntley, 2003). According to Lonneux *et al.* (1998a), potential weight gain is decreased up to 30 grams per day for every percentage of infested skin surface. This growth retardation can often not be recuperated by compensatory growth after treatment and can extend the fattening period (Lonneux *et al.*, 1998a; Rehbein *et al.*, 2003; Taylor *et al.*, 2007). Due to large areas of alopecia, the maintenance energy requirements of affected animals will increase and as thermal insulation is impaired, animals can be more susceptible to respiratory problems (Cole and Guillot, 1987; Tobin, 1962). Finally, in severe cases and mostly in young animals, the infestation may lead to the death of the host, due to dehydration, bacterial septicaemia or other causes (muscle deterioration, weakness, anorexia…). Together with the substantial treatment and prevention costs, these occasional mortalities can result in significant economic losses (Losson *et al.*, 1988; Millar *et al.*, 2011; Mitchell *et al.*, 2012; Pouplard and Detry, 1981; Taylor *et al.*, 2007; Wall, 2012).

Figure 5. Severely affected 11-month-old BB calf. The calf suffered from a generalized psoroptic infection, was anorectic, too small for its age and had skin that was unusable for hide production.

1.4 Immune response against mites

Little is known about the specific immune reactions that are induced during a *P. ovis* infection and it is still unclear whether these contribute to or counter the development of lesions. Previous research has suggested the former, as treatment of sheep with the anti-inflammatory drugs cyclosporin A and dexamethasone reduced lesion development (Bellworthy *et al.*, 1997; Huntley *et al.*, 2005). In the following chapter the immune response elicited by the host against *P. ovis* is described. In general, a pro-inflammatory and pro-allergic innate immune reaction develops within hours after the first contact with the mite. This pro-inflammatory response advances into a predominantly Th2-driven innate immune reaction with production of IgE and IgG antibodies. A comparison with immunologic data described for *S. scabiei*, the causative pathogen of scabies in dogs, pigs and humans, is also provided.

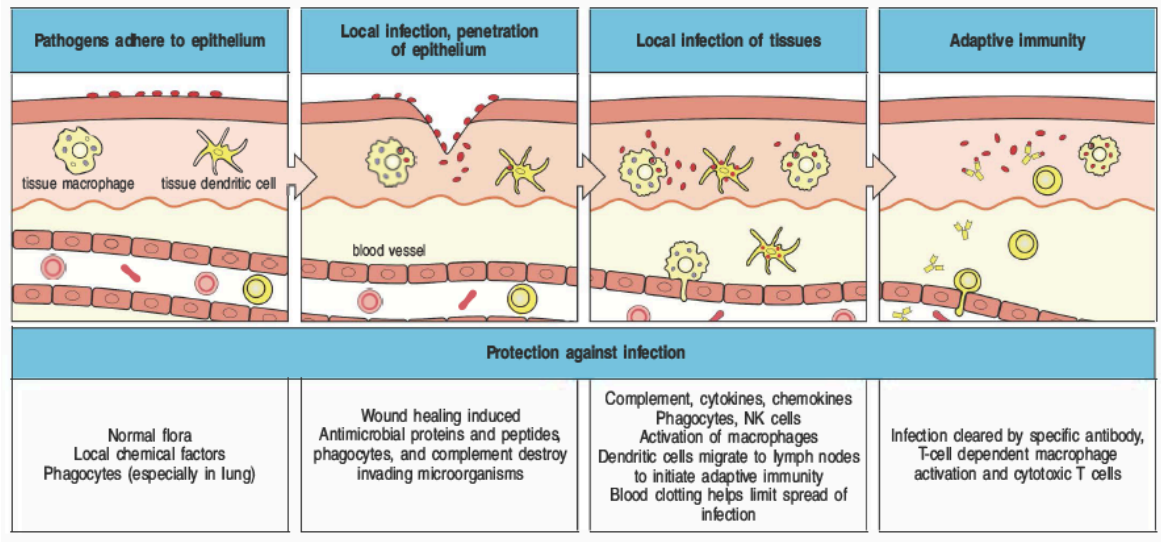
1.4.1 Introduction

The skin of healthy individuals is continuously challenged by pathogens and if these breach the skin barrier, most of them are eliminated quickly by the first line of defence, the innate immune system. At first, mainly soluble, preformed components, such as antimicrobial peptides or proteins from the complement system directly interact with the intruding pathogen. In a next phase, innate immune cells, such as macrophages, antigen-presenting cells (APC) and innate lymphoid cells (ILC) come in contact with non-self antigens. ILC are not T-cells but produce similar cytokine patterns and also include natural killer (NK)-cells. These innate T-cell counterparts have been demonstrated to be important in protecting the host against various infectious pathogens and play a role in wound healing (Hwang and McKenzie, 2013; Murphy, 2012). Specific pathogen-associated molecular patterns (PAMP) on the pathogen are recognized by innate immune cells using pattern recognition receptors (PRR) of which Toll-like receptors (TLR) are a subgroup. Hereupon, different signalling pathways and transcription factors are activated. This activation may lead to an attack on the intruding pathogen or to a general state of inflammation, but in both cases pro-inflammatory cytokines and chemotactic molecules are released (Murphy, 2012).

If an infectious agent is able to survive this first attack, the adaptive immune response is activated, which generates memory cells, inducing long-term and

pathogen-specific protection (Murphy, 2012). The adaptive immune reaction is characterized by 2 distinct phases: the cell-mediated and humoral immune response. First, naïve T-cells in the lymphoid organs recognize non-self antigens that are presented by circulating APCs. In response, these lymphocytes will differentiate, proliferate and become effector T-cells that activate macrophages and aid B-cells in producing antigen-specific antibodies. T-cells can specialize in different subsets, characterized by a specific cluster of differentiation (CD): cytotoxic CD8+ T-cells or CD4+ helper T (Th)-cells. Murine Th-cells are able to produce distinct cytokine profiles, marking them as Th1, Th2, Th17 or regulatory (Treg) cells. Whether bovine Th-cells subsets produce similar polarized cytokine patterns is still under discussion (Brown *et al.*, 1998). Besides this cellular immune response, there is also a humoral component, in which naïve B-cells are activated by APCs and the co-stimulatory signal from Th-cells. Activated B-cells, called plasma cells, produce antigen-specific antibodies that will neutralize the intruding pathogen or opsonize it for targeting by phagocytes. Th1-cells are thought to influence B-cells to produce IgG antibodies, while Th2-cells increase the allergic response by pushing plasma cells towards an IgE profile (Murphy, 2012). In Figure 6, a summary of the immune responses during infection is presented.

Figure 6. The different response stages during infection. When the local innate immune response fails to eliminate the infection, the adaptive immune response is induced by antigen-presentation between dendritic cells and naïve T-cells (© 2012 from Janeway's Immunobiology, Eighth Edition by Murphy. Reproduced by permission of Garland Science/Taylor & Francis Group LLC).



1.4.2 Innate immunity against Psoroptes ovis *in sheep and cattle*

The host immune response during the first hours of a *P. ovis* infection has been closely studied by Burgess *et al.* (2010) using transcriptomic analysis of skin biopsies from sheep. It should be stressed that as clinical signs in cattle are only noticeable after about one week, the immune responses in this species might also develop more slowly as compared to sheep (Stromberg and Fisher, 1986).

During the first contact between *P. ovis* and a suitable host, the mite deposits ES antigens on the host's skin, with keratinocytes being the first contact point. These antigens, including 'Pso o 1' and 'Pso o 3' (homologues of the house dust mite allergen groups 'Der p 1' and 'Der p 3') have a direct effect on epidermal cells because of their proteolytic activity. Moreover, they are able to cleave intercellular tight junctions and as such breach the epidermal barrier. Pathogens can either get directly attacked by the complement system or their PAMPs may be recognized by PRRs, such as TLRs (Nakamura *et al.*, 2006; Stoeckli *et al.*, 2013; van den Broek and Huntley, 2003; Wan *et al.*, 1999 and 2001). Several complement factors are down-regulated in ovine skin within 3 hours pi (Burgess *et al.*, 2010). Furthermore, the house dust mite allergen 'Der p 3' is known to cleave C3 and C5, 2 important parts of the complement system and this leads to the production of C3a and C5a (Maruo *et al.*, 1997). These pro-allergic peptides attract dendritic cells (DCs), eosinophils, neutrophils and mast cells and trigger their degranulation and as such already push the adaptive response towards a Th2 profile from early on (Arlian *et al.*, 1994b; Muller-Eberhard, 1988).

Besides being confronted with the complement system, pathogenic antigens can be recognized by TLRs on innate immune cells. Not only mite antigens, such as 'Pso o 2', the homologue of 'Der f 2', but also small amounts of bacterial lipopolysaccharide (LPS) are present on the skin of affected sheep and both molecules are recognized by TLR-4 together with the up-regulated, co-stimulatory molecule CD14 (Burgess *et al.*, 2010 and 2011). LPS might also be derived from the mites themselves as these often carry commensal bacteria. Through up-regulation of the adaptor protein myeloid differentiation (MYD) primary response gene 88, TLR-4 activates the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP-1) pathways. As a result, a pro-inflammatory Th2 driven adaptive immune response may follow, as has been described in helminth infections before (Burgess *et al.*, 2010, 2011 and 2012;

Kerepesi *et al.*, 2007). Beside the up-regulation of cyclooxygenase 2 (COX-2), an important inflammation mediator, some of the increased cutaneous pro-inflammatory cytokines that are released after NFκB pathway activation are tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1 and IL-8 (Burgess *et al.*, 2010 and 2011). IL-6 not only stimulates acute phase protein synthesis, it also induces T-cell activation, production of Th2 cytokines and proliferation of keratinocytes, which may lead to the typical hyperkeratotic scabs (Arlian *et al.*, 2003). Together with IL-6, suppressor of cytokine signalling 3 (SOCS-3) is activated, which has been linked to atopic dermatitis in humans as it regulates the onset of Th2-cells through the suppression of interferon (IFN)-γ production by keratinocytes (Seki *et al.*, 2003). The up-regulation of IL-4, IL-10 and IL-13 combined with the lack of Th1 cytokines IFN-γ and IL-18 in infested sheep skin around 6 hours post infection (pi) confirms the hypothesis that *P. ovis* most likely induces a Th2-driven immune response (Burgess *et al.*, 2010). The pro-inflammatory cytokine IL-1 is released by keratinocytes in response to skin damage and has been described as being important in human skin diseases (Debets *et al.*, 1995), mainly because it induces a number of pro-inflammatory chemokines. One of the earliest up-regulated chemokines is chemokine C-C motif ligand 26 (CCL-26), a specific eosinophil chemotactic molecule that could explain the early influx of eosinophils in affected tissue (Burgess *et al.*, 2011). IL-8 is another chemokine and together with TNF-α and complement factors C3a and C5a it mainly attracts neutrophils and eosinophils to the site of infection. *In vitro* cultured ovine keratinocytes transcribed high amounts of IL-8 mRNA within one hour after challenge with mite ES products (Erger and Casale, 1995; Watkins *et al.*, 2009). Other chemokines, such as CCL-2 and C-X-C ligand 2 (CXCL-2) are also up-regulated within 3 hours pi and mainly attract monocytes and neutrophils (Burgess *et al.*, 2010). Around 24 hours pi, these chemokines and cytokines, combined with the complement activation products increase the expression of cell-adhesion molecules, such as selectins, integrins and intercellular adhesion (ICAM) molecules on endothelial cells, potentially leading to a greater extravasation of lymphocytes and phagocytes, such as neutrophils, eosinophils and basophils to the site of tissue damage (Burgess *et al.*, 2010 and 2012).

During *P. ovis* infections in sheep, a fast influx of eosinophils, neutrophils, macrophages and lymphocytes in the infested skin is obvious within hours, which

correlates with a systemic lymphopenia. *In vitro* work demonstrated that specific mite derived factors that are part of mite ES material are responsible for a direct chemokinetic migration of ovine eosinophils (Wildblood and Jones, 2007) and the production of the pro-inflammatory chemokine IL-8 by ovine keratinocytes (Watkins *et al.*, 2009). This indicates that the mite might be directly responsible for the attraction of eosinophils and other immune cells in infested tissue *in vivo* and that the effects of these immune cells are partly beneficial for mite survival (Wildblood and Jones, 2007). Within days, an influx of mast cells is detected as well. Vascular permeability increases too, with oedema as a consequence (Murphy, 2012; Stromberg and Fisher, 1986; Stromberg *et al.*, 1986; Stromberg and Guillot, 1989; van den Broek *et al.*, 2000; van den Broek and Huntley, 2003; van den Broek *et al.*, 2004). The degranulation of mast cells and eosinophils may contribute to the damage of keratinocytes and the disruption of the epidermal barrier in general. Furthermore, the cytokines that are released during this process may guide the adaptive response in a certain direction. As mentioned above, one of those cytokines is IL-4: its release from mast cells and eosinophils is stimulated by specific mite allergens, such as 'Pso o 1' (van den Broek *et al.*, 2004) and it can direct the adaptive immune reaction towards a Th2-driven response. Other cytokines, such as IL-1, IL-8 and TNF-α will enhance the antigen presenting function of APC, furthermore linking the innate with the adaptive immune response (Sugita *et al.*, 2007). In sheep it was demonstrated that the early innate response at the level of the skin is augmented by the adaptive immune response, which results in a further influx of eosinophils, mast cells and other immune cells over time (van den Broek *et al.*, 2004). This was confirmed in circulating peripheral blood mononuclear cells (PBMC) in sheep that showed a peak up-regulation at 6 weeks pi of IL-4R and C-C chemokine receptor 3 (CCR-3), the receptor of CCL-26, which attracts eosinophils. Furthermore, pro-inflammatory molecules and peptides responsible for the extravasation of immune cells to the site of infection were still up-regulated after 3 to 6 weeks pi (Burgess *et al.*, 2012).

In order to counter the disruption of the epidermal barrier, mirrored by the down-regulation of several keratin and collagen genes in the skin of affected sheep, tissue repair mechanisms and keratinocyte differentiation genes are up-regulated within 24 hours pi. Important genes in this respect are members of the epidermal differentiation complex (EDC), such as small proline rich proteins (SPRR) and S100 calcium

binding proteins (Burgess *et al.*, 2010; Stoekcli *et al.*, 2013). EDC genes are responsible for maintaining the integrity of the epidermal barrier by coding for structural and regulatory proteins that are important for epidermal cornification and maturation of keratinocytes. The up-regulation of these proteins, most likely controlled by IL-1 and IL-13 (Zimmerman *et al.*, 2005), has been linked to allergy, psoriasis and atopic dermatitis in humans (Broome *et al.*, 2003; Sugiura *et al.*, 2005). S100 proteins have been shown to up-regulate IL-8 production and expression of ICAM-1, as well as promoting genes from the NFκB pathway (Viemann *et al.*, 2005; Vogl *et al.*, 2007). While these EDC genes are up-regulated, a clear down-regulation of some others (filaggrin, involucrin and loricrin) is observed within the same time frame, potentially caused by the up-regulation of transcription factor protein C-ets 1 (ETS-1) (Burgess *et al.*, 2011; Stoeckli *et al.*, 2013). Loricrin, involucrin and filaggrin can be down-regulated by the presence of IL-4 and IL-13, which are abundant in infested sheep skin (Burgess *et al.*, 2010; Howell *et al.*, 2007; Kim *et al.*, 2008). Furthermore, the *S. scabiei* homologue of the house dust mite serine protease 'Der p 3', has proven to actively digest filaggrin (Beckham *et al.*, 2009), with more skin barrier damage as a result. In addition, heritable loss of function mutations of filaggrin can cause several skin diseases in humans and could cause individual differences in susceptibility to mange in other species, such as cattle (Irvine, 2007; Stoeckli *et al.*, 2013). As described in humans with atopic dermatitis (Kim *et al.*, 2008; Sugiura *et al.*, 2005), suppression of EDC genes in general can accelerate the epidermal disruption causing excessive transdermal water loss but also an increased exposure to mite allergens. The latter can lead to continuous triggering of the immune response and more pronounced clinical signs (Burgess *et al.*, 2010; Stoeckli *et al.*, 2013). In circulating PBMCs, down-regulated levels of genes responsible for the skin barrier function maintenance were observed up till 3 weeks pi (Burgess *et al.*, 2012).

1.4.3 Adaptive immunity against Psoroptes ovis *in sheep and cattle*

When the innate immune response, which is not completely pathogen-specific and not protective in the long run, is insufficient to eliminate the pathogen, the adaptive immune response steps in. Increased *in vitro* peripheral blood T-lymphocyte reactivity towards mitogens and *P. ovis* antigen is documented in mange infested cattle, irrespective of the breed, and is linked to the extent of dermatitis and severity of the lesions (Lonneux *et al.*, 1998a; Losson *et al.*, 1988; Stromberg *et al.*, 1986).

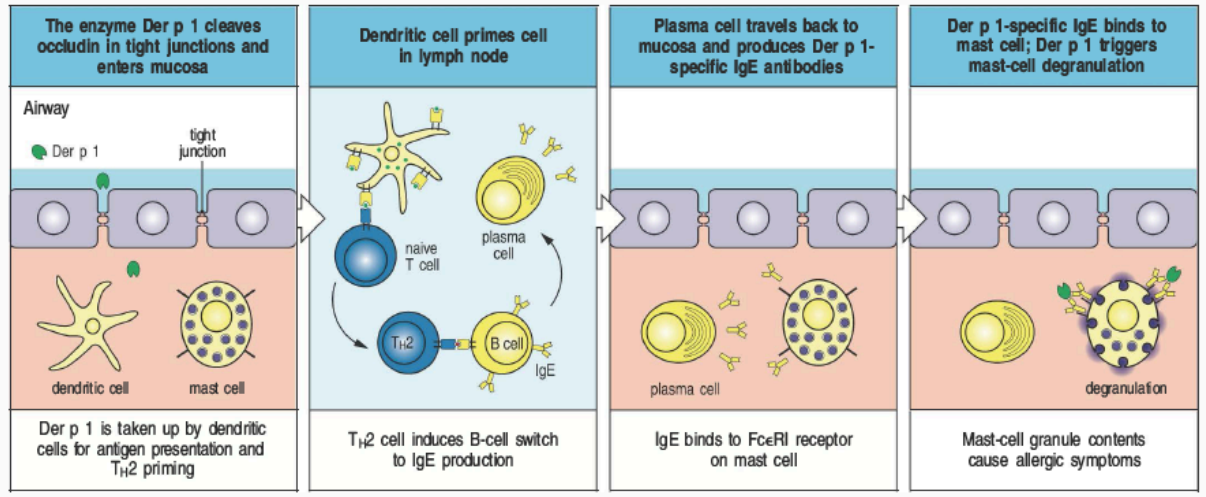
Research in sheep demonstrates higher PBMC proliferative activity in susceptible compared to more resistant breeds (Wildblood *et al.*, 2005). Several studies have described this increased lymphocyte reactivity: after primo-infection, *P. ovis* antigen-specific lymphocyte activity significantly increased between 7 to 40 days and returned to control values after 42 to 110 days, depending on the study. These observations indicate the presence of memory T-cells (Lonneux *et al.*, 1998b; Losson *et al.*, 1988; Pruett *et al.*, 1986). During subsequent challenge infections, contradictory observations were made as in one study the lymphocyte responsiveness increased sooner and was more pronounced but declined after about 7 weeks (Pruett *et al.*, 1986); while in another study the cell responsiveness occurred later, was less prominent and returned to control values already after about 4 weeks (Losson *et al.*, 1988). Nonetheless, if the mites are not cleared before the decline of lymphocyte reactivity, this could lead to a chronic and long-term stress reaction to the mites, with hyperplastic lymph nodes and adrenal glands as a consequence (Blutke *et al.*, 2015; Pruett *et al.*, 1986).

In the skin of infested sheep, a significant influx of CD4+ Th-cells and CD45RA+ B- or naïve T-cells can be measured 4 days after primo-infection. By day 8 pi, T-cell receptor γδ (TCRγδ) T-cells and CD1+ DCs also increase (van den Broek *et al.*, 2005). Next to CD4+, also substantially more CD8+, CD25+ and CD2+ circulating T-cells are detected in susceptible sheep breeds, while TCRγδ T-cells and CD14+ cells (macrophages and DCs) are mainly seen in resistant breeds (Wildblood *et al.*, 2005). Because of the eosinophil and mast cell infiltration in the skin of infested animals, CD4+ Th-cells are thought to produce an allergic Th2 cytokine pattern (van den Broek and Huntley, 2003; van den Broek *et al.*, 2005), which would correspond with the IgE-driven type 1 hypersensitivity reaction that is seen clinically (Figure 7). The presence of IL-4 that can be released by eosinophils and mast cells during the innate phase of infection contributes to this hypothesis, as it is known to direct naïve T-cells towards a Th2-cell polarisation (Murphy, 2012; van den Broek *et al.*, 2004). Up-regulation of IL-10 and IL-13 that suppresses macrophage function and stimulates B-cell differentiation and antibody production, further leads to a Th2 biased response (Figure 8) (Burgess *et al.*, 2010; Murphy, 2012). Similarly, TCRγδ T-cells are capable of producing Th2-related cytokines, as described during helminth infections (Ferrick *et al.*, 1995). The fact that these cells also express keratinocyte growth factor indicates a role in tissue repair and the attraction of more lymphocytes

to the site of epidermal damage (Boismenu *et al.*, 1996; Jameson *et al.*, 2002). The cutaneous B-cells may be responsible for local antibody production, but together with DCs they can also function as APC to increase T-cell activation (van den Broek *et al.*, 2005). While the number of TCRγδ T-cells seem to decline after 21 days, CD4+ Th-cells, B-cells (CD45RA+) and DCs numbers remain high till 49 days pi (van den Broek *et al.*, 2005).

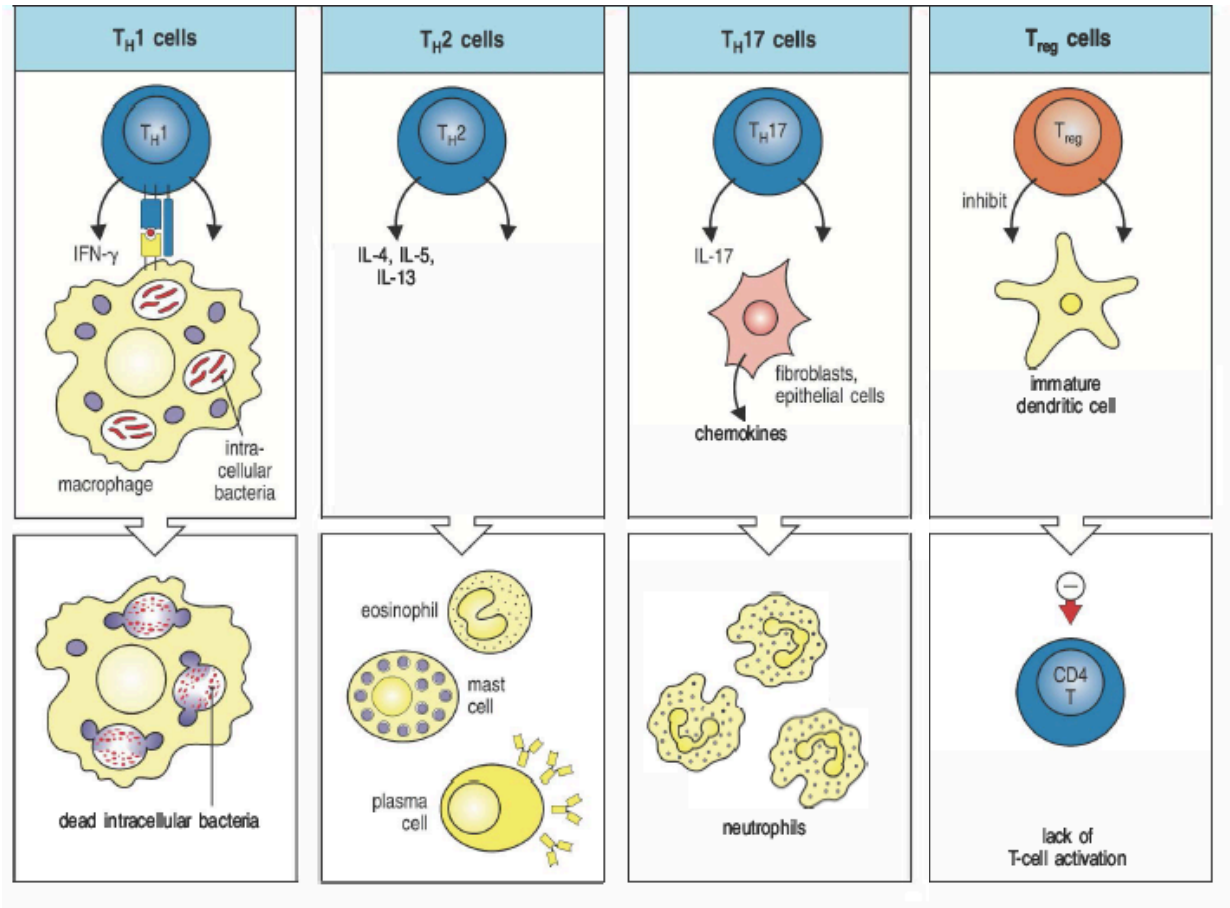
In order to modulate and counteract the (pro)-inflammatory effects of a Th2-driven hypersensitivity response, as soon as one day pi, Treg cell surface markers and transcription factors are up-regulated in the skin of infested sheep (Burgess *et al.*, 2010 and 2011). These cells, consisting of several subpopulations, suppress effector T-cell function, release regulatory cytokines, such as IL-10 and transforming growth factor (TGF)-β and can also have a direct cytotoxic effect on mast cells and eosinophils (Figure 8) (Askenasy *et al.*, 2008; Akdis, 2009). One of the Treg subpopulations are the forkhead box P3 (Foxp3)+ Treg-cells and by 2 weeks pi, significant numbers of these cells are detected in the skin of infested sheep (McNeilly *et al.*, 2010). These cells are typically CD4+ and CD25+ and, as mentioned above, higher numbers of these regulatory cells have been demonstrated in infested animals belonging to a susceptible sheep breed (Wildblood *et al.*, 2005). IL-10 producing Tr1 Treg-cells are another Treg-cell subpopulation and are believed to play a crucial role in controlling hypersensitivity reactions in humans (Hawrylowicz and O'Garra, 2005). However, the presence of these cells in bovine or ovine skin during *P. ovis* infections has not been demonstrated yet.

Figure 7. Allergic reaction caused by Der p 1, an inhaled allergen. Pso o 1, the Der p 1 homologue of *P. ovis* could elicit a similar reaction in the skin of infested animals (© 2012 from Janeway's Immunobiology, Eighth Edition by Murphy. Reproduced by permission of Garland Science/Taylor & Francis Group LLC).



Parallel to the onset of this cell-mediated immune response, the humoral immune response is initiated: *P. ovis* specific IgG antibodies are detectable in cattle as soon as one week pi (Lonneux *et al.*, 1996 and 1998b; Losson *et al.*, 1999) and in sheep from 2 weeks onwards (Bates, 1997; Nunn *et al.*, 2011; Ochs *et al.*, 2001; van den Broek *et al.*, 2003b). While no IgA antibody production is seen, IgM antibodies significantly increase 7 weeks pi in sheep (van den Broek *et al.*, 2003b). An increase in IgE antibodies in infested cattle is suspected to appear fairly late as only from 28 days pi the passive cutaneous anaphylaxis (PCA) test became positive, indicating the presence of IgE antibodies (Losson *et al.*, 1999). This is confirmed in sheep, in which IgE levels only start to rise after 6 to 7 weeks pi (van den Broek *et al.*, 2000). This late detection of IgE in cattle and sheep is in contrast with the assumption that this antibody plays a vital role from early on, as it is a component of the type 1 hypersensitivity reaction causing leakage of serum to the mite and causing the typical symptoms (Pruett *et al.*, 1986). However, IgE will mainly bind to mast cells in the skin, causing degranulation and inflammation, which means the functionality of IgE in the skin might precede the detection of IgE in the blood. This observation might explain the late detection of IgE in sheep and cattle using a serum enzyme-linked immunosorbent assay (ELISA) (Losson *et al.*, 1999; van den Broek *et al.*, 2000).

Figure 8. Subsets of CD4+ effector Th-cells. Th1-cells activate macrophages that are infested or have ingested pathogens, mainly through the release of IFN-γ. Th2-cells promote allergy and the immune response against parasites through B-cell activation and antibody (mainly IgE) production. Th17-like cytokines promote acute inflammation by attracting neutrophils and they support barrier integrity, including the skin. Treg-cells suppress T-cell activity (© 2012 from Janeway's Immunobiology, Eighth Edition by Murphy. Reproduced and adapted by permission of Garland Science/Taylor & Francis Group LLC).



After challenge infection, a rapid but moderate increase in IgG and high levels of IgE are documented in sheep from one week after re-infestation (van den Broek *et al.*, 2000 and 2003b), while no significant IgG production is seen in challenged cattle (Fisher, 1983b). However, the presence of IgE in re-infested cattle is suspected, as lesions occur very rapidly in a similar manner as observed in sheep (Stromberg and Fisher, 1986). Hence, for sheep and cattle it is hypothesized that a rapid increase in IgE during challenge infection is partly responsible for the rapid appearance of clinical signs but could also have a protective effect. The rapidly induced immediate type 1 hypersensitivity could facilitate the leakage of serum containing *P. ovis*-specific antibodies and complement factors to the mites, thus leading to the clearance

of the ectoparasite and the stagnation and eventual disappearance of clinical signs (Pruett *et al.*, 1998; Stromberg and Fisher, 1986; van den Broek *et al.*, 2000). Mites will take up antibodies while feeding (Pettit *et al.*, 2000) and the direct interaction between these antibodies and gut-associated proteins in the mite can impair digestion and lead to the death of the parasite. In addition, specific allergens in the mite's faecal pellets may be neutralized by these antibodies and as such counteract the induction of an allergic response in the host (Nisbet and Huntley, 2006). The presence of an IgE-driven type 1 immediate hypersensitivity can be confirmed with an intradermal skin test. *Psoroptes ovis* crude protein antigen is injected in the skin and will bind with mast cell-bound IgE. As a result, mast cells will degranulate and an immediate skin swelling can be observed after 15 minutes. Only in infested susceptible cattle (BB) a delayed skin response is seen after 48 to 72 hours (Losson *et al.*, 1988 and 1999) even though the underlying mechanism of this difference with the more resistant HF remains unclear. Infested sheep also display an immediate and delayed response after intradermal challenge, atypically characterized by the influx of eosinophils instead of mononuclear cells and neutrophils. This delayed influx of eosinophils is particularly noticeable in susceptible breeds (van den Broek *et al.*, 2003a; van den Broek and Huntley, 2003). The delayed skin reaction in infested BB and sheep could be identified as a Th2 mediated delayed eosinophilic hypersensitivity response, which has been described during other parasitic infections as well (Meeusen, 1999) and which could be a marker for breed susceptibility in both species (van den Broek *et al.*, 2003a).

1.4.4 Immunity during Sarcoptes scabiei *infections*

As in depth literature on the immunologic reactions against *P. ovis* is limited (Burgess *et al.*, 2010, 2011 and 2012; Stoeckli *et al.*, 2013), a comparison with the immune reactions during an infection with another mange mite, *S. scabiei*, is made in this chapter. Humans, cattle, pigs, dogs, camelids and several wildlife species (fox, wild boar…) are natural hosts for *S. scabiei* and for research purposes rabbits and mice often serve as alternative hosts (Taylor *et al.*, 2007). In humans, 2 clinical manifestations of scabies occur: common or ordinary scabies and crusted or Norwegian scabies. The latter mostly occurs in in developing countries or in socially disadvantaged communities of Indigenous populations where immune-compromised individuals are at higher risk. Immunosuppression can be linked to concurrent disease,

such as human immunodeficiency virus, leprosy or diabetes or to other health related factors, including alcohol abuse, malnutrition or overcrowding in low quality housing conditions. Crusted scabies is characterized by very high mite numbers and severe hyperkeratotic crusts, while people with ordinary scabies have less symptoms and lower mite burdens (La Vincente *et al.*, 2009; Roberts *et al.*, 2005; Walton *et al.*, 2004a and 2010). Similarly, different clinical appearances of *P. ovis* mange are seen in infested BB animals, making the underlying immune mechanisms of various scabies forms interesting to investigate in cattle as well. As the immune responses against *S. scabiei* are largely similar to those against *P. ovis*, only the clear differences will be reviewed in this chapter.

1.4.4.1 Innate immunity against *Sarcoptes scabiei*

The biggest difference between both mite species is that *P. ovis* lives superficially, while *S. scabiei* is a burrowing mite, which makes burrows in the epidermis of the host to deposit its eggs (Taylor *et al.*, 2007). Therefore, the epidermal barrier is immediately disrupted and mite ES products, including several house dust mite homologues (Arlian *et al.*, 1988), get in contact with the host immune cells, of which keratinocytes, skin DCs (Langerhans cells) and fibroblasts are the first (Arlian *et al.*, 2003). Mite allergens first encounter the innate complement system but specific scabies mite inactivated (serine) protease paralogs (SMIPPs) are known to inhibit this system in humans in order to evade the immune system (Bergstrom *et al.*, 2009; Fisher *et al.*, 2009; Holt *et al.*, 2003). These SMIPPs belong to the 'Sar s 3' antigens, *S. scabiei* specific homologues of the house dust mite 'Der p 3', and include scabies mite serpins (SMSs), serine proteases inhibitors of which 2 already proved to interfere with the human complement activation (Holt *et al.*, 2003; Mika *et al.*, 2012). By direct binding to several complement proteins (C1, C3, C4…) and through additive effects at the C9 level in the lectin pathway, the overall effect of these SMSs is the protection of the mite from complement-mediated gut damage (Mika *et al.*, 2012).

PAMP recognition by TLR on innate immune cells, such as DCs, also takes place and depending on the pathway that is activated, specific chemokines and cytokines are produced. Vascular endothelial growth factor (VEGF), responsible for increased vascular permeability and granulocyte-colony-stimulating factor (G-CSF), activator of neutrophil and DC formation are significantly up-regulated in keratinocytes and

fibroblasts after *in vitro* stimulation with *S. scabiei* extract, a phenomenon which is not seen during *P. ovis* infections (Arlian *et al.*, 2003). Whilst in the skin of *P. ovis* infested sheep, IL-6, IL-1, TNF-α, IL-8, IL-4, IL-13 and IL-10 are up-regulated, conflicting results are observed in humans after *in vitro* stimulation with *S. scabiei* extracts. Whereas IL-1 and IL-6 are up-regulated in dermal fibroblasts, keratinocytes and circulating PBMCs (Arlian *et al.*, 1996b, 2003 and 2004b; Walton *et al.*, 2008), IL-6 is down-regulated after stimulation of DCs and dermal endothelial cells (Arlian *et al.*, 2004b; Elder *et al.*, 2009). In parallel with the response during *P. ovis*, TNF-α production is also increased after *S. scabiei* stimulation of circulating PBMCs (Arlian *et al.*, 2004b). The production of IL-8 is up-regulated in dermal fibroblasts and PBMCs but no secretion is observed by keratinocytes, DCs and endothelial cells (Arlian *et al.*, 2003 and 2004b; Elder *et al.*, 2009). In addition, cell adhesion molecules selectin and vascular cell adhesion molecule 1 (VCAM-1) are suppressed in skin from humans with scabies, while they are up-regulated in *P. ovis* infested ovine skin (Elder *et al.*, 2006 and 2009). As these molecules, together with IL-8, are mainly responsible for the attraction and extravasation of immune cells to the site of infection, this can be an indication that *S. scabiei* tries to avoid the induction of inflammatory reactions, in that way delaying the display of clinical signs. The latter is also reflected in an up-regulation of anti-inflammatory TGF-β in the skin of humans and pigs with crusted scabies, an increased IL-10 production in circulating human PBMCs and the suppression of IL-6 production in human dermal endothelial cells (Arlian *et al.*, 2006; Mounsey *et al.*, 2015; Walton *et al.*, 2008 and 2010). Moreover, in the absence of IL-6, TGF-β can potentially induce the development of Treg-cells (McGeachy and Cua, 2008).

Despite several immune evasion strategies of the mite, chemotactic cytokines will attract a similar cell infiltrate as seen during *P. ovis* infections to the site of infection, with the composition of the cellular infiltrate depending on the host species (Arlian *et al.*, 1994b and 1997; Morsy and Gaafar, 1989; Mounsey *et al.*, 2015; Reunala et al, 1984; Stemmer *et al.*, 1996). Large numbers of eosinophils are particularly seen in humans affected by Norwegian scabies (Walton *et al.*, 2008).

1.4.4.2 Adaptive immunity against *Sarcoptes scabiei*

Circulating PBMCs from humans with scabies show a strong proliferation after *in vitro* stimulation with recombinant *S. scabiei* antigens and the effector cells that can

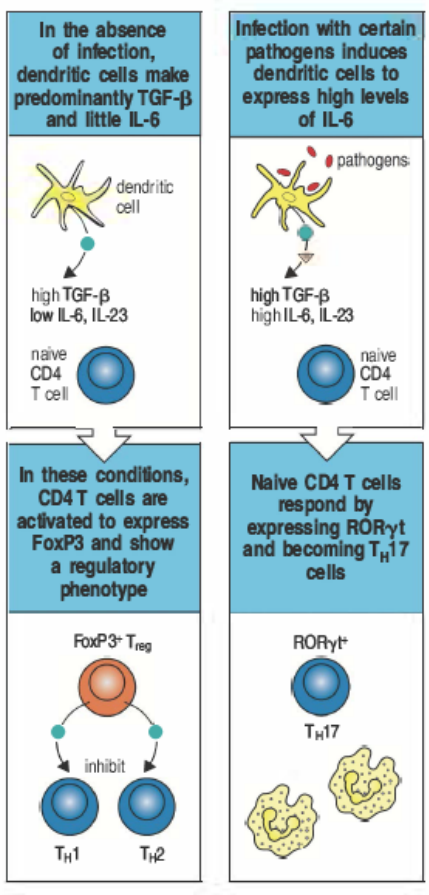
be found are mainly the same as during *P. ovis* infections (Walton *et al.*, 2010). In infested pigs, circulating CD4+ Th-cells and TCRγδ-cells proliferate from one week pi onwards (Liu *et al.*, 2014) and in infested humans, Treg-cells can also be found, although the function of these cells might be impaired (Arlian *et al.*, 2006; Walton *et al.*, 2008). The cell infiltrate in the skin of human patients with ordinary scabies predominantly consists of CD4+ T-cells, while in persons with Norwegian scabies and psoriasis mostly CD8+ T-cells are present in the skin, with almost no CD4+ T-cells (Bovenschen *et al.*, 2005; Cabrera *et al.*, 1993; Mounsey *et al.*, 2015; Reunala *et al.*, 1984; Stemmer *et al.*, 1996; Walton *et al.*, 2008; Wooten *et al.*, 1986). These findings correspond with data from pigs with crusted scabies, where CD8+, TCRγδ+ and IL-17 producing cells were the main cellular infiltrate in the skin (Liu *et al.*, 2014). Few to no B-cells are detected in humans with Norwegian scabies and this is in contrast to infested pigs, rabbits and humans with ordinary scabies (Arlian *et al.*, 1994b and 1997; Morsy and Gaafar, 1989; Reunala *et al.*, 1984; Walton *et al.*, 2008).

The cytokine profile that these immune cells produce has an influence on the clinical manifestation of the disease. Resistant dogs and rabbits display a more pronounced Th1 cell-mediated immune profile, while susceptible animals elicit a Th2-driven humoral reaction (Arlian *et al.*, 1994a and b, 1995 and 1996a). Similarly, *in vitro* re-stimulated PBMCs from pigs and humans with common scabies produce IFN-γ (Liu *et al.*, 2014), linked to a cellular Th1 response, while the cytokine profile in PBMCs from patients suffering from crusted scabies correlates with a humoral Th2 response with high levels of IL-5 and IL-13 (Walton *et al.*, 2004a and 2010). In porcine skin, higher levels of IL-4 and IL-13 are also present when animals suffer from the crusted form of the disease (Mounsey *et al.*, 2015). While these cytokine profiles are expected to originate from CD4+ T-cells, CD8+ T-cells, which are abundantly present in the skin of humans with Norwegian scabies, are able to do the same (Kelso and Groves, 1997). In addition, whereas lymph node cells from mice immunized with *Sarcoptes* extract, produce IFN-γ, lymph node cells mainly release IL-4 during *in vivo* primo-infection with *S. scabiei*. This observation indicates the living mite may induce molecules that inhibit IFN-γ production in favour of a Th2 response. However, when challenge infections were performed, these cells produced IFN-γ instead of IL-4, which suggests that protective immunity is linked with a Th1 cytokine profile (Lalli *et al.*, 2004). Finally, it is assumed that Th17 cytokines, such as IL-23 and especially IL-17, play an important role in the induction of crusted scabies

and other skin diseases, such as psoriasis. Pro-inflammatory IL-17 (Figure 9) can originate from mast cells, CD4+ Th2-cells and TCRγδ T-cells and the latter are present in high numbers in the skin of patients with Norwegian but not common scab (Liu *et al.*, 2014; Mounsey *et al.*, 2015; Stepanova *et al.*, 2012; Wang *et al.*, 2010). In general, a mixed Th2/Th17 cytokine profile might be typical for the development of severe clinical signs during crusted or hypersensitive scab infections (Mounsey *et al.*, 2015; Liu *et al.*, 2014).

There are contrasts between studies on antibody production with immunoglobulins increasing or decreasing during *S. scabiei* infection. The onset of antibody production after infection also seemed to differ according to the study (Walton, 2010). In general, increased levels of IgG, IgM and IgE are obvious from about 6 weeks pi in infested humans and pigs and this correlates well with the influx of B-cells. A correlation between antibody levels, including IgA and the extent of the lesions is also assumed, as during *P. ovis* infections (Morsy and Gaafar, 1989; Morsy *et al.*, 1993; Rampton *et al.*, 2013; Walton *et al.*, 2008 and 2010). IgE levels are especially high in human patients with crusted scabies. Nevertheless, the latter does not seem to lead to protection, which is in contrast to observations made in goats with sarcoptic mange (Arlian *et al.*, 2004a; Mounsey *et al.*, 2013; Tarigan and Huntley, 2005; Walton *et al.*, 2008 and 2010). As in sheep and cattle, IgE might be partially responsible for the development of clinical signs: after challenge infection, lower circulating antibody titres are seen in resistant rabbits compared to susceptible ones. Lower antibody titres are also linked with fewer plasma cells in the dermal infiltrate and it could confirm the hypothesis that in resistant animals a predominant cell-mediated Th1 and less of a humoral Th2 response is elicited (Arlian *et al.*, 1994a and b, 1995 and 1996a; Casais *et al.*, 2014; van den Broek *et al.*, 2000 and 2003a).

Figure 9. Dendritic cells produce cytokines that regulate the balance between Th17- and Treg-cell differentiation (© 2012 from Janeway's Immunobiology, Eighth Edition by Murphy. Reproduced by permission of Garland Science/Taylor & Francis Group LLC).



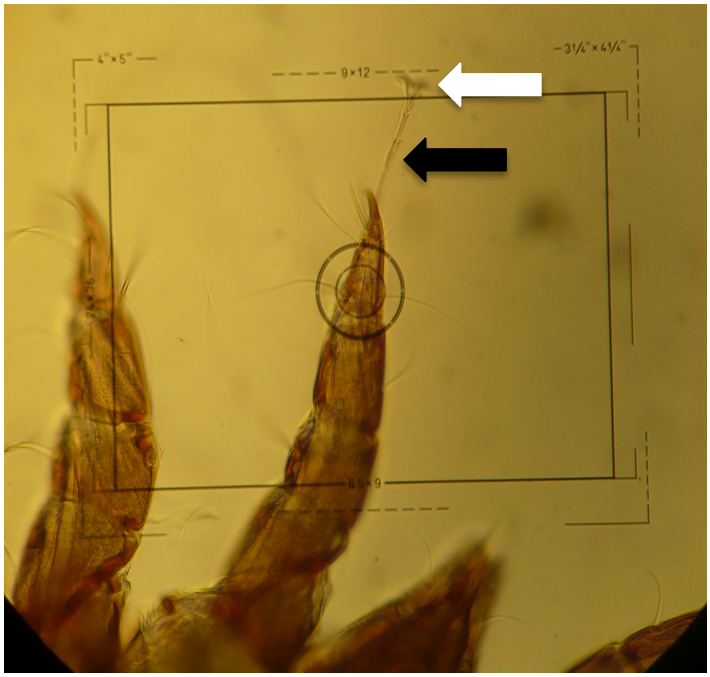
1.5 Diagnosis of psoroptic mange in cattle

Diagnosing psoroptic mange is relatively easy and it is a crucial step towards controlling the infestation. The typical clinical signs can be an indication that animals are infested: the presence of yellowish crusts and erythematous skin at the withers, back and tail base, secondary complications such as hematomas or abscesses, scratching, licking and restlessness due to pruritus, weight loss and a rapid spread of these symptoms towards other herd mates can suggest the presence of *P. ovis* mites on a farm (Bates, 1998; Pouplard *et al.*, 1990; Stromberg and Guillot, 1987). A differentiation from other ectoparasitic diseases, such as lice, *S. scabiei* or atypical *C. bovis* infections, mechanical skin damage (e.g. sacrum lesions from being mounted in oestrus) and other infectious diseases, such as bovine malignant catarrhal fever is however necessary as these can induce a comparable clinical image (Bates, 1997; Taylor *et al.*, 2007).

Psoroptic mange can be confirmed through the microscopic examination of skin scrapings, which are taken with a scalpel blade, superficially, at the edge of several lesions and examined as soon as possible (Lonneux and Losson, 1998; Pouplard *et al.*, 1990; Taylor *et al.*, 2007). Following facultative short exposure to a heat source to activate the ectoparasites, direct examination using a stereomicroscope (x25) for detection of live mites is essential (Lonneux and Losson, 1998; Pouplard and Detry, 1981; Pouplard *et al.*, 1990). Microscopic examination (Figure 10) will reveal the sharp mouthparts and the segmented pediculi and small pulvili, typical for *P. ovis* (Mitchell *et al.*, 2012; Soulsby, 1982). When direct examination is negative, indirect examination is performed after digesting the sample in 10% potassium hydroxide (KOH) overnight (Hollanders and Castryck, 1989; Lonneux and Losson, 1998; Mitchell *et al.*, 2012; Pouplard and Detry, 1981). This procedure will kill the mites and makes discrimination between live and dead mites impossible (Walton and Curie, 2007). The mites in the KOH suspension are enriched by centrifugation-flotation and microscopic examination of the concentrate is performed to identify the species (Hollanders and Castryck, 1989; Pouplard and Detry, 1981). *Psoroptes ovis* can be differentiated from other mange mites through microscopic investigation of the pediculi: *C. bovis* has very short, unsegmented pediculi that carry a large pulvillus (Figure 2) whereas the pediculi of *S. scabiei* are long but unsegmented (Figure 3) (Taylor *et al.*, 2007).

Examination of skin scrapings is a conventional way of diagnosing *P. ovis* infections and will directly confirm the presence of the mites, but there are some drawbacks: it requires expertise in ectoparasite identification, it can be time-consuming and it lacks sensitivity as subclinical or early infections are easily missed (Lonneux *et al.*, 1997a; Wells *et al.*, 2012). Finding mites in skin scrapings from subclinically infested carrier sheep by microscopic examination has been shown to have a probability of only 18% (Bates, 2009) and in pigs infested with *S. scabiei* as low as 10% (Smets and Vercruysse, 2000). Therefore, the development of improved, cost effective and precise diagnostic methods with higher sensitivity should be encouraged.

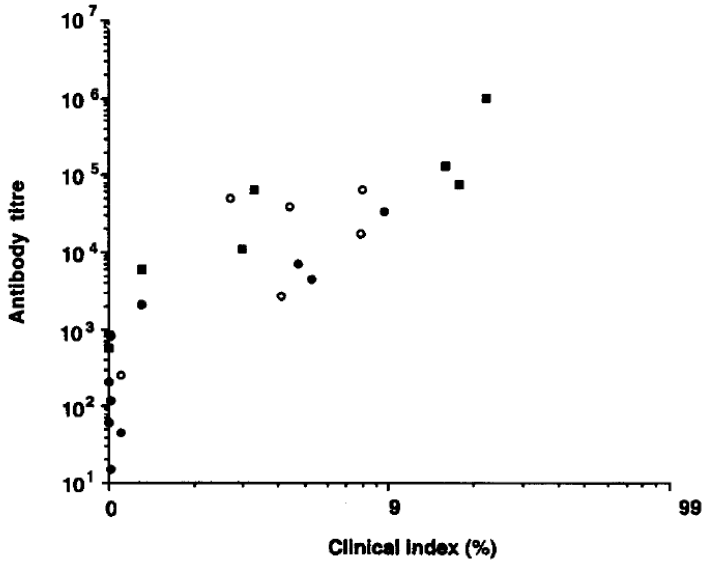
Figure 10. Close-up of the 3-jointed pretarsus or pediculus (black arrow) and pulvillus (white arrow) of *P. ovis* (x400).



One of those methods is the enzyme linked immunosorbent assay or ELISA, which is used to detect circulating anti-*P. ovis* antibodies in the blood of the infested host (Wells *et al.*, 2012). After confirming the presence of these specific antibodies in sheep and cattle (Fisher, 1972; Fisher and Wilson, 1977), an ELISA to detect them was developed in 1983 using crude *P. ovis* antigen (Fisher, 1983a and b). Nearly complete cross-reactivity between *P. ovis* and *P. cuniculi* could be demonstrated in 1991 (Boyce and Brown, 1991), which led to increased sensitivity of the bovine ELISA in 1996 by using *P. cuniculi* instead of *P. ovis* antigen. This measure decreased background values, as there was no contaminating bovine material present (Lonneux *et al.*, 1996). The ELISA revealed that antibodies in infested animals usually appear around the onset of the clinical signs (Fisher, 1983b), which can be as soon as one week after infection in cattle (Losson *et al.*, 1999). In sheep, small pathologic changes in the skin already occur within 24 hours pi, but ELISA often becomes positive after about 2 to 3 weeks when more obvious clinical signs are present (Bates, 1997; Nunn *et al.*, 2011; Ochs *et al.*, 2001). Antibody titres in both host species correlate with mite numbers and the degree of dermatitis (Figure 11) (Pruett *et al.*, 1986; Lonneux *et al.*, 1998a). Although still under discussion in sheep, as mentioned above, in cattle, clinical signs are usually obvious at the same time or even before the ELISA turns positive, which reduces the practical value of this

technique. Moreover, mostly crude proteins are used but identification of the key antigens that induce antibodies and production of recombinant versions of those antigens may further improve the sensitivity, specificity, reproducibility and cost. Indeed, an ELISA using the recombinant antigen 'Pso o 2', a house dust mite homologue, has proven to be an excellent tool to diagnose psoroptic mange in sheep. Antibodies can be detected as soon as 2 weeks pi and even seem to be able to identify subclinical carrier animals (Burgess *et al.*, 2012; Nunn *et al.*, 2011). A similar highly sensitive ELISA is an IgE ELISA using the specific recombinant mite allergen 'Sar s 14.3' to diagnose *S. scabiei* affected humans (Jayaraj *et al.*, 2011). Other downsides of this diagnostic tool are the potential cross-reactivity with C. bovis (Jacober *et al.*, 2006) and the fact that an ELISA is usually performed only once in the field. Hence, it is impossible to distinguish circulating antibodies during active infection from residual antibodies from a previous infestation (Wells *et al.*, 2012). Although anti-*P. ovis* antibodies start declining from 7 to 14 days after treatment, in parallel with the disappearance of the mites, they may persist in the blood up till 6 months after effective treatment in sheep and up till 7 months in cattle, causing a bias for the interpretation of the ELISA results (Bates, 2009; Burgess *et al.*, 2012; Lonneux *et al.*, 1997a).

Figure 11. Correlation between the extent of dermatitis (clinical index, %) and the antibody titre (logarithmic scale) in cattle. Data from trial 1 (circles) and trial 2 (squares) on day 56 post-infection show a positive correlation between the degree of dermatitis and the antibody titres measured by sandwich ELISA (from Lonneux *et al.*, 1996 with permission of Elsevier).



Other diagnostic tools could therefore be of help. A pen-side lateral flow assay that is currently being developed for psoroptic mange provides not only improved diagnostic accuracy in the field, but also reduced costs, leading to an easy, precise and complete assessment of *P. ovis* infections on farm level. This efficient microfluidic technique resembles a modified sandwich ELISA, as capillary membrane bound mite specific antibodies are used to detect mite antigens in the field. A similar test is available to detect lice infections in New Zealand by using wool fibres (Bates, 2009; Wells *et al.*, 2012). Additional diagnostic tools that not only indicate (previous) contact with the pathogen, but also give an indication of the phase of infection (subclinical, active, chronic or healing) are often based on host-parasite interactions but are currently not used to diagnose psoroptic mange in the field (Wells *et al.*, 2012). Examples of such immunodiagnostic techniques are the identification of key signalling transcription factors or blood biomarkers, such as specific acute phase proteins (haptoglobin, serum amyloid A, complement 4 binding protein-β chain) indicating active or early infections (Wells *et al.*, 2012 and 2013a and b). The latter has already been used in diagnosing psoroptic mange in sheep (Wells *et al.*, 2013a and b) and sarcoptic mange in Alpine ibex (Rahman *et al.*, 2010). Molecular methods, such as polymerase chain reactions (PCR) are already used to experimentally detect *Demodex canis* DNA in canine hair plucks and skin biopsies (Ravera *et al.*, 2011).

1.6 Treatment and prevention of psoroptic mange

1.6.1 Treatment of psoroptic mange

Treating psoroptic mange is not an easy task and several aspects should be taken into consideration to achieve complete eradication (O'Brien, 1999). As cattle may be affected by several mange mite species and/or other infectious diseases that may induce similar clinical signs, a diagnosis to confirm the presence of *P. ovis* should be performed first and an appropriate acaricide should be chosen accordingly (O'Brien, 1999; Taylor *et al.*, 2007). It should be emphasized that 100% efficacy for any acaricide is desirable because only a couple of surviving mites can cause new symptoms and a rapid spread of the disease in a herd (Bates *et al.*, 1995).

Leaving out products such as sulphur and mercury, the first commonly used drugs to treat psoroptic mange were organochlorines (lindane) in the 1940's,

organophospates (diazinon, coumaphos, propetamphos and phoxim) since the 50's and synthetic pyretroids (flumethrin, deltamethrin and cypermethrin) from the 70's onwards, all of them used for plunge dipping (sheep) or as pour-on's (Losson, 1997; O'Brien, 1999; Taylor *et al.*, 2007; van den Broek and Huntley, 2003). A double topical administration of amitraz or flumethrin with an interval of 10 days has proven to be completely effective and is currently still used to treat psoroptic mange in cattle (Guillot *et al.*, 1982/1983; Harrison and Palmer, 1981; Losson and Lonneux, 1992). Successful topical treatment is encouraged when most of the crusts are removed before treatment and the animals are thoroughly wetted (Lonneux and Losson, 1996; Plant, 2006). Although phoxim is still used for treating mange in pigs, it is not registered for cattle in Belgium. Most of these products lack a persistent effect, may induce considerable side effects and are toxic for the environment, so nowadays they are pushed aside by the group of macrocyclic lactones (ML) in the treatment of psoroptic mange. This group consists of the avermectins (ivermectin, eprinomectin and doramectin) and the milbemycins (moxidectin) and they are available in injectable, pour-on and oral formulations. MLs interact with the glutamate-gated chloride channels (GluCls) in arthropods and nematodes and as such increase nerve cell membrane permeability to chloride ions, inducing paralysis and often death of the parasite. Γ-aminobutyric acid (GABA)-gated chloride channels may be targeted by MLs as well (Vercruysse and Rew, 2002). These drugs are safe as GluCls typically only occur in invertebrates. Furthermore, these products offer a broad-spectrum activity against endo- and ectoparasites, they are easy to use and numerous cheaper generic formulations have been developed over the years (Losson, 1997; O'Brien, 1999; Pouplard and Detry, 1981; Sallovitz *et al.*, 2002). Ivermectin was the first ML to prove it's beneficial effect in the treatment of psoroptic mange and both doramectin and moxidectin followed quickly.

In cattle, all of these drugs gave similar results: one injection at a dose of 0.2 milligram (mg)/ kilogram (kg) body weight (BW) led to the elimination of the infection after 14 to 28 days (Barth, 1980; Clymer *et al.*, 1997; Guillot and Meleney, 1982; Logan *et al.*, 1993; Lonneux *et al.*, 1997b; Lonneux and Losson, 1992; Pouplard and Detry, 1981; Wright and Guillot, 1984). Although animals were not parasitologically examined between day 0 and day 7 after treatment, one study demonstrated a 100% efficacy of ivermectin after 7 days (Lonneux *et al.*, 1997b). This means that directly after and up till 7 to 14 days post-ML-treatment, live and

infective mites may still be present on treated individuals. Therefore, treated animals should be isolated from the rest of the herd for at least one week (O'Brien, 1999).

In sheep, single ML injections gave more variable results compared to cattle, but it should be stressed that treatment of sheep is often more challenging in general. The presence of wool or fleece may impede the application of topical drugs and as *P. ovis* mites on sheep do not ingest serum, systemically applied products can have an inferior effect, leading to the need for multiple treatments (Kirkwood, 1985; Ortega-Mora *et al.*, 1998). Indeed, single ivermectin injections were not always 100% sufficient (Bates and Groves, 1991), but by treating the animals 2 or 3 times with an interval of one week or with an intraruminal controlled-release formulation, complete efficacy was reached (Benz *et al.*, 1989; Forbes *et al.*, 1999; Sargison *et al.*, 1995; Soll *et al.*, 1992). For doramectin, a 93% efficacy was observed after injecting 0.2 mg/kg BW and only the 0.3 mg/kg BW dose gave a 100% efficacy after 7 days (Bates *et al.*, 1995). For moxidectin, although mite counts and lesions were significantly reduced, both a single and double injection failed to completely control the infection; the maximum efficacy was 97% and >90% respectively after 56 days (Ortega-Mora *et al.*, 1998).

Beside the selection of a suitable acaricide, some other factors should be taken into consideration in the treatment of psoroptic mange in cattle. The life cycle and epidemiology of *P. ovis* give an indication of some of these essential steps: as the duration of the cycle is fairly short (about 10 days) and the disease is highly contagious, it spreads fast. Moreover, subclinical carriers are often present. These factors indicate the necessity to treat all in-contact animals at the same time (Jones *et al.*, 2014; Mitchell *et al.*, 2012; Taylor *et al.*, 2007).

Secondly, the thick yellow crusts enclose large amounts of mite eggs, they protect the mites from dehydrating and prevent good contact with topical acaricides, therefore it is advised to shear the animals and remove the largest crusts before treatment. Injectable formulations are distributed to the infection site through the blood and the acaricidal activity will be achieved at the level of the skin. By shearing the animals before treatment, all mites that are located further away from the skin, i.e. on the hairs or in crusts, are removed, which will benefit the treatment efficacy (Jones *et al.*, 2014; Lonneux and Losson, 1996; Plant, 2006).

In addition, none of the available products has a complete ovicidal effect (Lekimme *et al.*, 2006a; Lonneux and Losson, 1996) therefore, products with no persistent efficacy, such as amitraz and flumethrin should be administered at least twice. The second treatment is applied after the eggs have hatched but before these immature stages become sexually active (and produce eggs of their own), which makes an interval of 7 to 10 days ideal (Lekimme *et al.*, 2006a; Lonneux and Losson, 1996; Taylor *et al.*, 2007). One injection of MLs, which should have a prolonged efficacy due to their lipophilic nature, used to be sufficient to control a *P. ovis* infection (Barth, 1980; Clymer *et al.*, 1997; Guillot and Meleney, 1982; Logan *et al.*, 1993; Lonneux *et al.*, 1997b; Lonneux and Losson, 1992; Pouplard and Detry, 1981; Wright and Guillot, 1984), but since a couple of decades this seems to be inefficient (Benz *et al.*, 1989; Clymer *et al.*, 1997; Lonneux and Losson, 1996; Pouplard and Detry, 1981; Vercruysse and Rew, 2002; Vercruysse *et al.*, 2008). Therefore, at least 2 treatments are recommended for any product, especially for the treatment of BB cattle (Jones *et al.*, 2014; Lonneux *et al.*, 1997b; Minihan *et al.*, 2002; Mitchell *et al.*, 2012; O'Brien, 1999; Vercruysse *et al.*, 2008; Wall, 2012). To avoid having to treat twice, the application of long acting (LA) formulations could be beneficial, in which a highly concentrated ML is administered and released slowly over time. Such products have shown their persistent efficacy in several gastro-intestinal endoparasite infections (Rehbein *et al.*, 2015; Sargison *et al.*, 2012; Yazwinski *et al.*, 2006), but the efficacy against psoroptic mange remains unclear. While a single injection of ivermectin LA (3.15%) as a prophylactic treatment in cattle was 100% efficacious from 56 days before, till 35 days after challenge infection (Bridi *et al.*, 2001), infested animals that were therapeutically treated only became parasitologically negative after 2 to 4 weeks (Blutke *et al.*, 2015; Hamel *et al.*, 2015; Rehbein *et al.*, 2002). A single injection of moxidectin LA (10%) gave various results: although one study demonstrated that BB animals remained mange free for 77 days (Losson *et al.*, 2008), a recent study showed that in the field this procedure was not sufficient to control the infection (Mitchell *et al.*, 2012). The use of the LA formulation of moxidectin (2%) against sarcoptic mange in sheep showed that animals were protected against infection up till 60 days, but in infested animals complete clinical cure was only reached 40 days after single injection. It must be stressed that no mite counts were performed in this study and efficacy was based purely on the evaluation of the clinical symptoms (Astiz *et al.*, 2011). Finally, a single extended-release injection of

eprinomectin in cattle with sarcoptic mange eliminated all mites by 1 to 4 weeks after treatment (Visser et al., 2013). These observations indicate that animals treated with MLs, including LA formulations, are still infective for some days post-treatment.

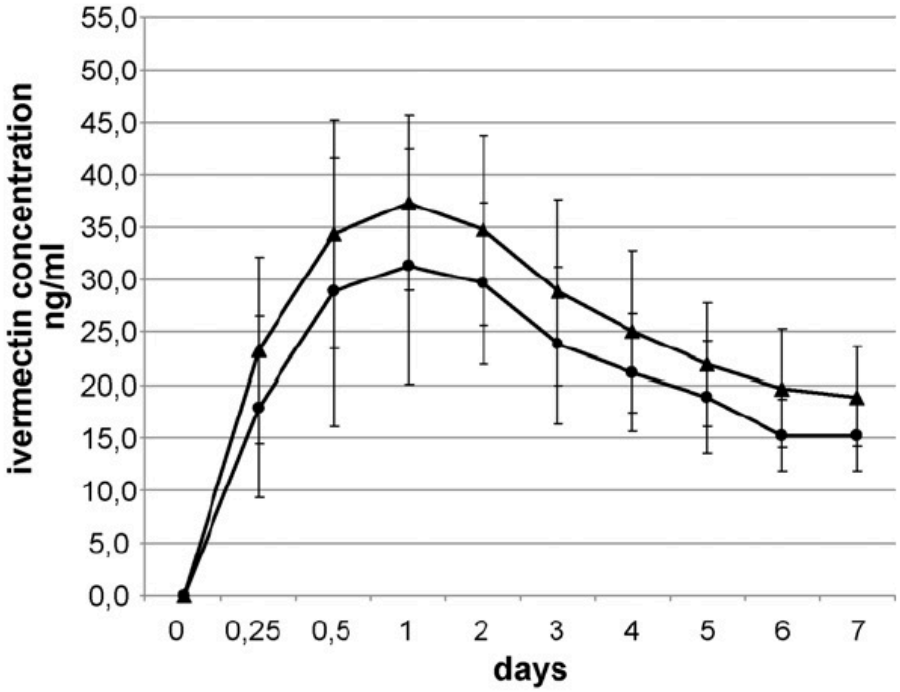
Finally, the mite's feeding behaviour will influence the optimal administration route of acaricides: as cattle specific *P. ovis* feeds on cell debris, lipids but also serum, systemically applied (injectable) MLs at 0.2 mg/kg BW have the largest beneficial effect (Lonneux and Losson, 1996; Mitchell *et al.*, 2012; Taylor *et al.*, 2007). Orally administered MLs have an inferior effect and should not be used to treat mange (Vercruysse and Rew, 2002). Although successful results have been described in the past (Lonneux and Losson, 1992; Losson and Lonneux, 1996), pour-on MLs (at 0.5 mg/kg BW) are not recommended as these have proven to be less efficacious against *P. ovis* compared to injectable formulations (Benz *et al.*, 1989; Lonneux *et al.*, 1997b; Millar *et al.*, 2011; Mitchell *et al.*, 2012; Vercruysse and Rew, 2002). After pour-on application, large differences in plasma concentrations and percutaneous absorption have been documented depending on the cattle breed (Sallovitz *et al.*, 2002) and pharmacokinetics of pour-on formulations may vary depending on the product of choice. In parallel with findings on subcutaneously administered doramectin and ivermectin, it was demonstrated that the area under the plasma concentration-time curve and the mean residence/persistency time of doramectin were higher than those of ivermectin and that the inter-individual variation in plasma concentration was less (Gayrard *et al.*, 1999). A potential explanation for this individual variability comes from previous research that demonstrated that substantial amounts of a pour-on formulation are taken up orally through licking or grooming behaviour and as such have a substantial anti-parasitic effect. However, the degree of this individual oral uptake and the exposure levels of the drug in the blood are highly variable (Bousquet-Mélou *et al.*, 2011). In conclusion, pour-on application of MLs may lead to highly variable exposure doses in the host and as such, some individuals may be over-exposed, while others are under-exposed. Not only can this lead to therapy failure on individual and herd level, it can also stimulate the development of acaricide resistance in the mites (Bousquet-Mélou *et al.*, 2011). In exceptional cases pour-on formulations may be justified, for instance when treating animals becomes difficult when they are on pasture or when they become unmanageable after multiple injections. For the treatment of adult dairy cattle on the other hand, the only available products are eprinomectin and moxidectin pour-on at 0.5 mg/kg BW. Moxidectin should be

preferred, as eprinomectin officially has no claim for *P. ovis* (Barth *et al.*, 1997; Mitchel *et al.*, 2012; Taylor *et al.*, 2007).

1.6.2 Treatment failure

Although treatment failure is often reported in the field, few detailed scientific studies on the possible reasons for insufficient treatment efficacy are available. Potential causes of treatment failure in general could be failing to identify the mite species, misapplication of the acaricide (inappropriate formulation or incorrect interval between treatments, only treating clinically affected animals, not shearing prior to treatment) or individual variances in the pharmacokinetic of the applied drug (Plant, 2006; Wall, 2012). In BB for instance, higher plasma and skin levels of ivermectin could be documented after a single subcutaneous injection compared to HF animals. Potential explanations could be inherent differences in skin physiology, body or carcass composition and nutritional status (Sallovitz *et al.*, 2002; Vercruysse *et al.*, 2008). Lastly, therapy failure could be caused by the presence of resistant mites (Plant, 2006; Wall, 2012). For *S. scabiei*, several *in vitro* tests have been developed and resistance has already been identified in humans (Brimer *et al.*, 1993; Currie *et al.*, 2004; Mounsey *et al.*, 2009; Nong *et al.*, 2014). The frequent but often incorrect use of MLs causes high selection pressure and potential resistance in the mites (Lekimme *et al.*, 2010; van den Broek and Huntley, 2003). There are indications of *P. ovis* resistance in the field, as 3 ivermectin treatments failed to eliminate the infestation in a group of affected BB animals (Lekimme *et al.*, 2010; Losson, 1997). It should be stressed that in this study a 14 days interval was used between treatments, when 7 to 10 days are advised. Furthermore, a generic formulation of ivermectin was used and considerable differences in kinetic behaviour and therapeutic efficacy between generics and the original formulation have been demonstrated (Figure 12) (Genchi *et al.*, 2008; Lifschitz *et al.*, 2004). Finally, *in vitro* confirmation of apparent *in vivo* resistance is impossible as no *in vitro* tests for assessment of acaricide resistance in *P. ovis* are available yet. To avoid the development of acaricide resistance, animals should not be under-dosed, LA products should be used with care and products of choice should be regularly alternated (Losson, 1997).

Figure 12. Mean concentration of ivermectin reference formulation (triangles) and ivermectin generic formulation (circles) in cattle (from Genchi *et al.*, 2008 with permission of Elsevier).



1.6.3 Prevention of psoroptic mange

Beside therapeutic treatment, general management aspects should be taken into account to control and/or prevent *P. ovis* infections at farm level. Introduction of new untreated herd members, which may be subclinical carriers, is one of the main sources of infection on a farm and quarantine measurements should therefore always be implemented after purchase of new animals (Carty and Nisbet, 2011; Millar *et al.*, 2011; Mitchell *et al.*, 2012; Phythian *et al.*, 2013; Pouplard *et al.*, 1990; Taylor *et al.*, 2007). Contact between treated and untreated animals should be avoided and it is advised to leave cleaned pens, where infested animals were previously housed, unoccupied for 14 to 21 days, as this will dry out and kill any remaining mites (Bates, 1998 and 2012; Losson, 2003; O'Brien, 1999; van den Broek and Huntley, 2003). Off the host, *P. ovis* mites remain infective up till 2 weeks. Therefore, general hygiene protocols, such as frequently changing the bedding and cleaning tools should be implemented in order to minimize re-infection from the environment (Lonneux and Losson, 1996; Mitchell *et al.*, 2012; O'Brien, 1999; Pouplard and Detry, 1981).

Furthermore, attention should be given to optimizing the feed composition, amount and quality and on controlling concomitant infectious diseases in order to guarantee an optimal herd immunity (Minihan *et al.*, 2002; O'Brien, 1999; Phythian *et al.*, 2014; Pouplard and Detry, 1981).

1.6.4 Alternative treatment methods

In the long run, upcoming resistance and residues in meat, milk and the environment will only further increase the need for alternative treatment methods (Pruett, 1999b; Smith *et al.*, 2001). Preliminary research in the field of microclimate manipulation and biological control agents has not delivered useful treatment tools yet (O'Brien, 1999; Smith *et al.*, 2001; van den Broek and Huntley, 2003). However, some fungi, such as *Beauveria bassiana*, have proven to have an effect on *P. ovis* as mites were killed, egg hatchability was decreased and the life span of emerging larvae was reduced after exposure to fungal conidia (Lekimme *et al.*, 2006b and 2008). In addition, antibiotic treatment with the aim of eliminating the symbiotic bacteria *Comamonas* spp. in the mite gut, led to decreased *P. ovis* survival (Hall *et al.*, 2015). Furthermore, some natural substances, such as petroleum ether extract, lavender, tea tree and neem oil have been tested and seem to have some acaricidic efficacy (Nong *et al.*, 2014; Perrucci *et al.*, 1994; Walton *et al.*, 2000; Walton *et al.*, 2004b). Future research is necessary to further explore the possibilities and practicality of these alternative control strategies.

Another alternative option in treating mange could be the selection of resistant cattle, which might reduce or even disregard the need for chemical treatments as a whole, but this is still far out of reach at this moment (Bates, 2012; O'Brien, 1999; Pruett, 1999b; Smith *et al.*, 2001).

Another interesting alternative treatment method is vaccination. As previously affected cattle and sheep seem to develop a fast increase in *P. ovis* specific antibody titres and show less severe lesions during challenge infections, the development of a protective immunity or resistance, potentially able to get boosted by vaccination, is suspected (Bates, 2000; Pruett *et al.*, 1986; Stromberg and Fisher, 1986). Moreover, mites digest immunoglobulins that are released in the serous exudate during feeding, bringing them in contact with the host's defence mechanism (Smith *et al.*, 2002). As the immunopathogenesis of the disease is not fully clarified yet, it remains difficult to select specific antigens that could potentially function as vaccine candidates (van den

Broek and Huntley, 2003). Immunization of rabbits with a whole mite extract of *P. cuniculi* induced partial protection (Uhlír, 1992). Similarly, vaccination of sheep with fractions from crude *P. ovis* extracts increased specific antibody titres and reduced mite numbers, lesion areas and pathology in general (Smith *et al.*, 2002; Jayawardena *et al.*, 2000; Stella *et al.*, 1997) and remarkably all these antigens seemed to be water-soluble (Smith *et al.*, 2001). Several purification and filtration procedures were able to concentrate one of those fractions to 2 proteins of 20 to 22 kilodalton (kDa) with more pronounced protective effects as a consequence. However, these allergens could not be exactly identified (Smith *et al.*, 2002; Smith and Petit, 2004). In the search for more specific and potentially recombinant allergens, several possible vaccine candidates have been put forward, some of them linked to the feeding and digestion behaviour of the mites (McNair *et al.*, 2010). Most of them are homologues of house dust mite allergens. A partially purified soluble extract of *P. ovis* was used to immunize cattle by Pruett *et al.* (1998). Although the results were not statistically significant, 8 out of 14 animals were free of lesions around 2 months after challenge infection. The increased grooming activity in response to a pruritic immediate allergic reaction that was elicited in the vaccinated animals was believed to play a role in protection (Pruett *et al.*, 1998). This partially pure fraction was further purified in 1999 and a 16 kDa antigen, homologous with the 'Der f group 2' allergens from *Dermatophagoides farinae*, could be identified as 'Pso o 2' (Pruett, 1999a). A *Dermatophagoides pteronyssinus* 'Der p group 1' homologue has been identified in *P. ovis* by Lee *et al.* in 2002, called 'Pso o 1'. This allergen appeared to be a cysteine proteinase and provoked an immediate skin reaction and dramatic rise in specific IgG antibodies in sheep that were previously exposed to mites. Nevertheless, the reaction was less pronounced compared to the whole-mite extract and vaccination studies were not performed (van den Broek *et al.*, 2003a). The fact that in humans, inhibition of 'Der p 1' activity interrupted the development of the allergic Th2 reaction and reduced the development of severe symptoms, indicates an important role for this allergen (John *et al.*, 2000). 'Der p 10' (tropomyosin), 'Der p 11' (paramyosin) and 'Der p 14' (vitellogenin/apoliopohorin) were the next immunodominant allergen homologues that were identified in *P. ovis* (Huntley *et al.*, 2004). Even though these homologues had variable results in vaccination trials against other parasites, further investigation is needed to assess the effect against *P. ovis* (Nisbet *et al.*, 2006). Vaccination is not yet applied as a control measure to prevent psoroptic mange and

although it looks as if vaccination will never be able to fully protect individuals from getting infested through induction of sterile immunity, it certainly has potential to reduce parasite counts and clinical disease (Pruett, 1999b). Moreover, mathematical modelling demonstrated that in the field a 100 % efficacy in all animals might not be necessary for conventional vaccines to have a beneficial effect on disease severity and spread of infection (Barnes *et al.*, 1995). For example, the recombinant Bm86 vaccine against *Boophilus microplus* significantly reduces larval abundance instead of protecting individual cattle. Nevertheless, the use led to a decrease in acaricidic treatments and in the incidence of bovine babesiosis (Vercruysse *et al.*, 2007).

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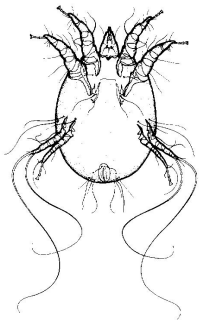


OBJECTIVES

Objectives

Psoroptes ovis is a ubiquitous ectoparasite that causes mange in livestock. Clinically, an allergic dermatitis with wet crusts, alopecia and excoriation is observed at the withers, back and tail base. Differences in susceptibility to psoroptic mange are obvious within and between cattle breeds. While Holstein Friesian (HF) animals are more resistant to the disease, Belgian Blue (BB) cattle seem to be hypersensitive with severe and often generalized lesions as a consequence. Within this cattle breed a variety in sensitivity is also noticeable on group and individual level, but the mechanisms behind this observation remain unclear. Physical differences, such as a distinctive skin conformation or the presence or absence of horns and behavioural varieties have been suggested as possible influencing factors. In sheep, the wool composition, bacterial skin flora and the influence of farm management have been identified as potentially important and in this species the ability to mount an allergic immune response during infection also appeared to have an effect on the clinical outcome. Whether these factors are also important in cattle remains uncertain.

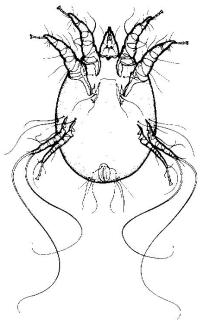
The overall aim of this research project was therefore to identify potential causes of the susceptibility differences within the BB cattle breed and between the BB and HF breed. Two potentially influential factors, the environment and the host itself, led to 2 specific research questions.

1. **Do farm related management factors, including treatment protocol, influence the differences in susceptibility to mange that are observed between individual BB farms?**

   A risk analysis was performed to identify potential risk factors for mange on farm level (Chapter 2) and 2 treatment protocols were evaluated on 9 mange problem farms in order to assess the influence of an (in)correct treatment schedule on the persistence of psoroptic mange on a farm (Chapter 3).

2. **Do individual immunologic host factors explain the susceptibility of the BB breed to psoroptic mange, especially when compared to the HF breed?**

   The *in vivo* cutaneous and *in vitro* cellular immune responses in uninfested and infested BB were evaluated and compared to those in HF animals (Chapter 4).



CHAPTER 2

Risk factors for *Psoroptes ovis* mange on Belgian Blue farms in Northern Belgium

Based on: C. Sarre, K. De Bleecker, P. Deprez, B. Levecke, J. Charlier, J. Vercruysse, E. Claerebout. (2012) Risk factors for *Psoroptes ovis* mange on Belgian Blue farms in Northern Belgium. Veterinary Parasitology 190, 216-221.

2.1 Introduction

The mite *Psoroptes ovis (P. ovis)* is a common ectoparasite of cattle. It causes a severe exudative dermatitis, characterized by thick scabby lesions and an intense pruritus (Stromberg and Guillot, 1989). As these pathological changes have a significant effect on the animal's metabolism and its growth, mange can lead to important economic losses, especially in the beef industry (Cole and Guillot, 1987; Lonneux *et al.*, 1998; Rehbein *et al.*, 2003).

The geographical distribution of the parasite in cattle is limited to certain areas, such as continental Europe and some parts of the USA (Guillot, 1984; Clymer *et al.*, 1997; Losson *et al.*, 1999; Minihan *et al.*, 2002; Lekimme *et al.*, 2010). Remarkably, psoroptic mange is only a major issue in some beef breeds, such as the Belgian Blue (BB) breed, whereas in dairy cattle (*e.g.* Holstein Friesian (HF)) clinical symptoms are rare (Lonneux *et al.*, 1998; Losson *et al.*, 1999; Rehbein *et al.*, 2003; VLA Disease Surveillance Report, 2010). Besides the differences between breeds, mange problems can vary substantially between farms and even between individual barns on a farm, suggesting that management factors, such as acaricide treatments, feed, housing and barn climate may be important. This hypothesis has also been suggested in sheep. A study performed on sheep farms in Great Britain demonstrated that several farms within high-risk areas for mange had never experienced problems with mange. This indicates that an appropriate management can overcome the higher risk of disease (Rose *et al.*, 2009). Despite the importance of cattle mange in several countries, no unambiguous data on the prevalence of the disease are available.

In Belgium, psoroptic mange is common, but recent information about the prevalence is lacking as well. The BB breed represents the biggest part of the Belgian beef production and this breed is highly susceptible to psoroptic mange. In contrast to HF, the disease in BB often takes on a chronic, generalized form and repeated treatments may give disappointing results (Minihan *et al.*, 2002; Losson, 2003; VLA Disease Surveillance Report, 2010; Lekimme *et al.*, 2010). Moreover, outbreaks of psoroptic mange in other countries are often initiated by the import of a BB animal (Minihan *et al.*, 2002; Mitchell, 2010; VLA Disease Surveillance Report, 2010). In accordance with the hypothesis of Rose *et al.* (2009), mange problems often appear to vary significantly between cattle farms in the field, but the potential effect of specific farm practices on the outcome of the disease in cattle has never been described.

Therefore, the objectives of this chapter were two-fold: (1) to estimate the prevalence and clinical importance of psoroptic mange in beef herds in Northern Belgium (Flanders) and (2) to identify potential risk factors for mange on farm level.

2.2 Materials and methods

2.2.1 Cross-sectional questionnaire survey

A questionnaire was developed and tested in a pilot study on 4 farms to evaluate the validity of the approach and the comprehensibility. The questionnaire contained questions about the severity of mange on the farm and the farm management, with emphasis on acaricide treatments.

After fine-tuning, the questionnaire was sent to 1,800 farms in Flanders. The farms were selected from Sanitel, which is the Belgian central database for the identification and registration of animals (http://www.favv.be/dierlijkeproductie/dieren/sanitel/). The only inclusion criterion was the presence of at least 20 Belgian Blue animals over 24 months of age. Consequently, both beef farms and mixed beef and dairy farms were included in the study. A random number generator in Excel was used to select the farms.

2.2.2 Farm visits

Ten percent of the farms with a completed questionnaire were visited to validate the questionnaire and to retrieve additional information on specific management parameters. Based on the questionnaire results, the farms were divided into 2 groups: farms with significant mange problems that were difficult to control or uncontrollable and farms without mange problems or with mange problems that were easy to control during the previous winter. Within these 2 groups, the farms were selected by a random sampling method (random number generator in Excel), stratified by province and weighed according to the response rate in each province. In total 66 farms were visited, *i.e.* 36 farms with severe mange problems and 30 farms with little or no mange problems. During the farm visit, the farmer was interviewed, using the same questions as in the questionnaire survey (Annex 1). Feed labels were collected to get detailed information about the different feed components. All barns were inspected to evaluate the floor type, ventilation system, barn orientation, number of places at the feeding rack, straw management and animal stocking rate where ≥ 1 m^2

per 100 kilograms of body weight is acceptable (Annex 2). A general hygiene score (0 – 4) was given to each barn based on a hygiene score for dairy cattle (Hulsen, 2004). Light intensity (lux) and relative humidity (%) in the barn and temperature (°C) in and outside the barn were determined using a Testo 540 light intensity meter and a Testo 610 humidity and temperature meter. For cattle, the optimal light intensity is 150 lux or more, the temperature comfort zone is between 5°C and 15°C and relative humidity should not be higher than 80% (reference values in Nantier, 2006).

The number of animals with skin lesions compatible with mange was counted per barn and a scratching index (ScI) was calculated to estimate the intensity of the mange problem. The scratching index was obtained by observing 10 animals per barn for 5 minutes. Each licking or scratching action was counted and the total number of actions was then divided by the number of observed animals. Skin scrapings were taken from 5 animals with signs of mange to determine the mite species present. The scrapings were examined through direct and indirect microscopic examination (after 10% KOH digestion followed by sedimentation-flotation with sucrose) (Damriyasa *et al.*, 2004). A clinical index (CI, % affected body surface) was calculated for the same animals by marking and photographing the affected body areas. The photos were analysed using the software program ImageJ in order to obtain a CI for each animal. An arithmetic mean CI was determined per barn.

Serum samples were taken from the jugular vein of 6 clinically healthy animals between 9 and 12 months of age. In these samples, copper (Cu) and iron (Fe) concentrations were determined with a Gallery™ automated photometric analyser (Thermofisher) and zinc (Zn) and selenium (Se) levels were quantified by graphite furnace atomic absorption spectrophotometry (GF-AAS) (Oster and Prellwitz, 1982).,

2.2.3 Statistical analysis

The concordance between answers in the questionnaire and results from the farm visits was determined using a Kappa test. Kappa-values higher than 0.75 indicate an excellent agreement. Values between 0.4 and 0.75 demonstrate a good agreement and values between 0 and 0.4 suggest a marginal agreement (Rosner, 2000).

Only data from herds for which a fully completed questionnaire was available were used in the risk analyses. The questionnaire and farm visit data were analysed by a logistic regression model to evaluate the associations between herd management

factors (independent variables, Annex 1 and 2) and the presence of mange (dependent variable) (SPSS Statistics 19). There were 4 possible dependent variables available (see (*) in Annex 1 and 2) and the dependent variable used in this analysis was 'no mange problem' (absence of mange or mange easy to control) versus 'severe to uncontrollable mange problem'. Initially a univariate binary logistic regression was performed to identify potential risk factors. In the univariate analysis a significance level $P < 0.2$ was used to reduce the risk of unintentionally overlooking putative risk factors. Significant parameters were subsequently tested for correlations through Chi Square tests (χ^2), as all variables were categorical. When a significant association was detected between 2 parameters (level of significance $P < 0.05$) only one of the 2 parameters was selected for further evaluation. The remaining parameters were ultimately combined in a backward stepwise multivariate analysis ($P < 0.05$). Presence of confounders in the final multivariate model was tested by comparing the regression coefficients of the remaining variables when one variable was excluded.

The farm visit data were collected from multiple barns per farm. To account for clustering of observations within farms, a random effect for herd was included in the logistic model of the farm visits (MLwiN v2.19). Statistical significances for fixed parameters were based on Wald tests ($P < 0.05$). However, as no significant effects were found in this cluster analysis, only the results of the logistic regression model are shown.

2.3 Results

2.3.1 Questionnaire validation

Information was obtained from 680 of the 1,800 selected herds (38%). The mean response rate per question was 36% with a range from 31 to 38%. A fully completed questionnaire was obtained from more than half of these farms (n = 351, 52%). In total 238 barns on 66 farms were visited. The results of the Kappa test demonstrated a good concordance (0.4 – 0.75 or >0.75) between the questionnaire results and the responses obtained during the farm visits.

2.3.2 Prevalence of mange

Mange was present on 74% (95%CI (70.7 – 77.3)) of all farms. Although on most of these farms less than 20% of the animals were infested, approximately half of

the farmers perceived the mange problem on their farm as difficult to control or uncontrollable (Questionnaire (Q): 44% – Farm visits (FV): 53%). On most farms, the susceptible breed was the BB breed (Q: 83% - FV: 98%). The skin scrapings confirmed that *P. ovis* was present in 58% of the sampled barns, with or without other ectoparasites, such as *C. bovis* or lice. Despite the high prevalence of psoroptic mange, only in a small proportion of the barns (14%) the scratching index was high (>1) and in most of the sampled animals (80%) the affected body surface was less than 10%. Details are listed in Annex 1 and 2.

2.3.3 Applied management procedures

Most farmers provided concentrate to the animals (FV: 73% – Q: 88%) and approximately half of them provided additional mineral supplements (FV: 41% – Q: 52%). Nevertheless, low serum Cu and Se levels were found in 60% and 46% of the sampled animals, respectively.

Most animals were housed on a full floor with straw bedding (88%). Straw was replaced at least weekly on most farms (73%) and hygiene scores of the animals were generally good (72%). At the time of the farm visits, temperature in most barns was within or below the comfort zone for cattle (73%) and relative humidity was below 80% on 86% of the barns, but 53% of the barns were too dark (<150 lux). Animals were housed in groups on 73% of the farms, and here stocking rates were usually acceptable (55%), but physical contact between groups of animals was possible (84%).

On most farms, the cattle received an acaricide treatment at housing (Q: 69% – FV: 99%). Most of the animals were shorn prior to treatment (Q: 88% – FV: 91%) and were treated with macrocyclic lactones (Q: 36% – FV: 51%, mostly as a pour-on formulation) or amitraz (FV: 36% – Q: 44%). In most cases all animals were treated (Q: 79% – FV: 93%), but less than half of the farmers treated the animals twice or more with an interval of ≤ 10 days (FV: 24% – Q: 46%). On the majority of the farms (Q: 49% – FV: 97%), additional mange treatments were given during the housing period. These additional treatments were similar to the treatments at housing, except that almost half of the farmers (FV: 29% – Q: 49%) only treated clinically affected animals and that shearing prior to treatment was applied less frequently (FV: 20% – Q: 69%).

Further details of the descriptive statistics are presented in Annex 1 and 2. Annex 1 shows the descriptive statistics of the parameters that were addressed in both the questionnaire and the farm visits. Annex 2 illustrates the descriptive statistics of the additional parameters that could only be measured during the farm visits.

2.3.4 Risk analyses

2.3.4.1 Cross-sectional questionnaire survey

The univariate analysis (results not shown) revealed a high number of potential risk factors, such as the period of the year (autumn/winter *vs*. spring/summer *vs.* year round), additional mineral supplements in the feed and almost all parameters concerning acaricide treatments. All these parameters were positively associated with the presence of mange. Because the χ^2 tests revealed that all treatment related parameters were significantly correlated with each other (results not shown), only one treatment parameter 'mange treatments' was used in the multivariate analysis together with 'period of the year' and 'supplements'. The results of the multivariate analysis (Table 1) indicated that mange problems occurred more often with an increasing number of acaricide treatments. In most cases mange problems were perceived the whole year round. When this was not the case, mange was more present in autumn/winter in comparison with spring/summer.

Table 1. Significant parameters in the multivariate binary logistic regression of the questionnaire survey data ($\alpha < 0.05$).

Variable (Levels)	**Odds Ratio**	**95% CI**	***p*-value**
Period			<0.001
Spring-summer	Baseline	1	-
Autumn-winter	2.211	(0.719; 6.797)	0.166
Year round	6.492	(1.955; 21.562)	0.002
Mange treatments			<0.001
None	Baseline	1	-
Only HT	2.920	(1.440; 5.921)	0.003
Only AT	3.109	(1.270; 7.608)	0.013
HT and AT	9.815	(4.781; 20.152)	<0.001

2.3.4.2 Farm visits

The univariate analysis (results not shown) indicated a fairly high number of significant parameters. Mange appeared to be a problem all year round on most farms. The disease was also more prevalent in beef farms in comparison to mixed beef-dairy farms, when mineral supplements were fed to the animals and when a higher temperature in the barn was measured. On the other hand, problems with mange occurred less frequently when a sufficient number of places at the feeding rack was provided and when the animals had a good hygiene score. In addition, significant effects were found for the animals purchase policy, the availability of (supplemented) concentrate, the separation between (groups of) animals, the frequency of straw bedding replacement, the ventilation system and the relative humidity. In contrast to the questionnaire analysis, the overall treatment parameter 'mange treatments' was not significant and could therefore not be used in the multivariate analysis.

Because many parameters were correlated according to the χ^2 tests (results not shown) the number of parameters to include in the multivariate analysis was reduced. Concerning the barn climate, the ventilation system was chosen together with the animal hygiene score, which was preferred over the frequency of bedding replacement. Both of these parameters, together with the other significant parameters from the univariate analysis (see above) were included in the multivariate logistic regression.

This multivariate analysis revealed that mange was found less in cleaner cattle (higher hygiene score) and that on most farms mange was present during the whole year. Significant effects were also identified for the purchase policy and the feeding of (supplemented) concentrate (Table 2).

Table 2. Significant parameters in the multivariate binary logistic regression of the farm visit results ($\alpha < 0.05$).

Variable (Levels)	Odds Ratio	95% CI	*p*-value
Purchase policy			0.001
0	Baseline	1	-
1-5	0.686	(0.342; 1.379)	0.290
5-15	3.472	(0.908; 13.276)	0.069
>15	3.004	(1.309; 6.893)	0.009
Period			<0.001
Year round	Baseline	1	-
Autumn-winter	0.145	(0.070; 0.302)	<0.001
Spring-summer	0.000	(0.000; ∝)	0.999
Hygiene score			
Unclean	Baseline	1	-
Clean	0.477	(0.239; 0.952)	0.036
Concentrate			0.032
No	Baseline	1	-
Yes	1.608	(0.627; 4.125)	0.323
Yes (supplemented)	0.562	(0.281; 1.124)	0.103

2.4 Discussion

A questionnaire survey and subsequent farm visits were performed in this chapter to estimate the prevalence and clinical importance of psoroptic mange in BB cattle in Flanders and to identify putative risk factors. There was a good concordance between the response obtained by the questionnaire and the farm visits, except for some questions about mange treatments. The discrepancy can be explained by the fact that the treatment schedule was thoroughly discussed during the farm visits, whilst farmers often gave vague and incomplete answers to the same questions asked in the questionnaire. These were also the only open questions, which makes interpretation of the answers more difficult. Still, it can be concluded that the questionnaire and farm visit answers were sufficiently correlated to consider the survey analyses results as trustworthy.

Results of the skin scrapings demonstrated that *P. ovis* is the most common ectoparasite on Flemish beef or mixed dairy-beef farms. The perceived herd prevalence of mange in Flanders was 74% (95%CI (70.7 – 77.3)), which confirms the

ubiquity of this disease in northern Belgium. Concerning the affected proportion of the herd, there was a difference between the farmers' perception and our observations during the farm visits. Although most farmers recognised the presence of mange on their farm, the majority (86%) estimated that less than 20% of their animals were infested. In contrast, the farm visits results demonstrated that on 61% of the farms more than 20% of the animals were found visibly affected, suggesting that a number of farmers underestimated the mange problem in their herd. Although a high percentage of herds and animals are infested with *P. ovis*, the scratching index in most barns was lower than one and the majority of the animals had a CI below 10%, which indicates that most infections had a relatively mild character.

Surprisingly, the risk analyses demonstrated a significant positive association between all variables related to acaricide treatment and the presence of mange. Probably this suggests that farmers that are aware of mange problems on their farm treat more frequently and more intensively. This study indicated that, taking into consideration all treatment parameters, less than half of the farmers applied an optimal treatment strategy at housing. When additional treatments were applied in winter, compliance with advised treatment strategies was even poorer. In other animal species, such as goats and camelids, it has also been reported that farmers often apply an incorrect treatment scheme (Lusat *et al.*, 2009). Farmers and veterinarians should be informed about the importance of a correct diagnosis of psoroptic mange, including identification of the mite species, followed by a correct acaricide treatment and follow-up of the treatment efficacy.

The questionnaire and farms visit results in this chapter demonstrated that mange was a problem all year round on most farms. On farms where this was not the case, the questionnaire results demonstrated that mange was significantly more present in autumn/winter than in spring and summer. This seasonality was also observed in goats in the UK, but not in alpaca's (Lusat *et al.*, 2009). Possible reasons for a higher incidence of mange during the housing season may be close contact between the animals and favourable environmental conditions (cold, humid) for the off-host survival of mites (Smith *et al.*, 1999). Although higher indoor temperatures were positively related to mange in the univariate analysis of the farm visit data, this factor was not retained in the multivariate analysis. Results of humidity were inconclusive.

The hygiene score of the animals had a negative association with the occurrence of mange. A possible explanation is that faeces and urine that remain on the animal can affect the skin barrier, making an animal more susceptible for developing clinical symptoms. This parameter was also strongly associated with the straw management on the farm (χ^2 = 16.040, *P*-value = 0.003). The risk of having mange was the lowest on farms in which the boxes were cleaned daily. In pigs, the negative effect of straw bedding (*vs*. strawless) on mange was also demonstrated (Damriyasa *et al.*, 2004). Although direct contact between animals is most likely the main source of infection (Hourrigan, 1979; Minihan *et al.*, 2002; Losson, 2003), this finding suggests that transmission of mites from the infested environment to the animals might be an important factor in the epidemiology of mange and should not be neglected.

Purchasing animals also appeared to be a risk factor for mange. Lusat *et al.* (2009) reported that the import of new animals and a large herd size were positively associated with mange in goats and camelids. In buffaloes, it was also demonstrated that the presence of other animals and importation are related with mange problems (El-Khodery *et al.*, 2010). However, according to the farm visit analyses, purchasing 1 to 5 animals had a significant negative association with mange. It should be stressed that this category was mainly represented by farmers who bought only one bull, which was housed separately on the farm and often also treated against mange before being introduced in the herd. The risk of having mange after purchasing one bull is therefore much smaller than after the purchase of multiple animals that are immediately housed together with the rest of the herd. In general, purchased animals should be treated and kept in quarantine for 2 to 3 weeks, to avoid the introduction of the infection in the herd (Lusat *et al.*, 2009).

Although many farmers provided concentrate and/or mineral supplements to their animals, the majority of the sampled animals showed a deficiency in Cu and Se. Low Se levels appear to be a pervasive problem in the BB breed (Guyot *et al.*, 2009). Cu has an important role in the immune system and Cu deficiency may affect the progress of a mange infection (Guyot *et al.*, 2009). The cause of the low Cu levels remains unclear. However, no conclusive results were obtained about possible associations between feeding of minerals or (supplemented) concentrate and the risk for mange.

In conclusion, mange is highly prevalent in BB cattle in Flanders (74%), although the clinical appearance of the disease is rarely severe. Because the disease induces important economic losses and intense pruritus, it can be considered as an important threat and animal welfare issue for the Belgian beef industry. The 13% of farms that did not experience any mange problems in BB cattle demonstrate that not only the susceptibility of the breed is important in the aetiology of mange, but also farm management parameters. This chapter revealed that differences in hygienic measures and purchase of animals can explain some of the variation in mange problems across farms. It was also demonstrated that infections with mites from the environment should not be neglected. Together with a correct treatment scheme, these management factors could help the farmer and veterinarian to control mange on the farm.

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2.6 Annex 1

Descriptive statistics of the questionnaire (Q) and farm visit (FV) results, with the proportion of herds and the corresponding kappa-values.

Variable (Levels)	Description	Q	FV	Kappa-value *(95%CI)*
		N (Proportion%)	*N (Proportion %)*	
Herd information				
Farm type				1 (1; 1)
Beef	The farm was a pure beef farm	439 (65)	154 (65)	
Mixed	The farm was a mixed beef and dairy farm	241 (35)	84 (35)	
Problem breed				1 (1; 1)
BB	BB is the breed with the most mange problems	544 (83)	234 (98)	
BB without problems	BB on the farm without mange problems	87 (13)	0 (0)	
No BB	No BB present on the farm	29 (4)	4 (2)	
Purchase policy	Number of purchased animals per year			0.827 (0.72; 0.94)
0		177 (26)	71 (30)	
1-5		285 (42)	98 (41)	
6-15		95 (14)	17 (7)	
>15		119 (18)	52 (22)	
Mange problem				
Infested animals*	Percentage of infested animals during the past 3 months			0.078 (-0.08; 0.24)
0%		177 (26)	39 (16)	
1-20%		407 (60)	55 (23)	
>20%		96 (14)	144 (61)	
Perception problem*	Perception of mange problem by the farmer			1 (1; 1)
No problem		114 (17)	26 (11)	
Easy controllable		267 (39)	86 (36)	
Difficult to control		281 (42)	81 (34)	
Uncontrollable		14 (2)	45 (19)	
Period	Period of the year when mange is most common			1 (1; 1)
Spring-summer		36 (6)	2 (1)	
Fall-winter		407 (69)	168 (70)	
All year round		146 (25)	68 (29)	
Spread	The spread of the lesions on most animals			1 (1; 1)
Localised		492 (87)	190 (80)	
Generalised		72 (13)	48 (20)	
Feed				
Concentrate				1 (1; 1)
No	Animals do not get concentrate in the feed	76 (12)	65 (27)	
Yes, always	Animals always get concentrate in the feed	351 (54)	162 (68)	
Yes, fattening	Animals only get concentrate in the feed in the fattening period	218 (34)	11 (5)	
Supplements				0.650 (0.46; 0.84)
No	Animals do not get extra mineral supplements	313 (48)	140 (59)	
Yes, always	Animals always get extra mineral supplements	302 (47)	98 (41)	
Yes, fattening	Animals only get extra mineral supplements in the fattening period	30 (5)	0 (0)	
Acaricide treatments				
Mange treatments				0.129 (-0.001; 0.26
None	No mange treatments	132 (20)	0 (0)	
Only HT	Only treatment at housing	195 (30)	8 (3)	
Only AT	Only additional treatments during winter	74 (12)	2 (1)	
HT and AT	Mange treatment at housing and additional mange treatments	247 (38)	228 (96)	

*possible dependent variable

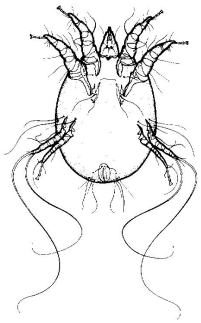
2.7 Annex 2

Descriptive statistics of the farm related parameters investigated in all 238 visited barns (during 66 farm visits).

Variable (Levels)	Description	*N (Proportion%)*
Mange problem		
Scratch index*	Scratch index per barn	
0		57 (24)
0-1		148 (62)
1-2		28 (12)
2-3		5 (2)
Clinical index*	Clinical index per barn	
0-1%		16 (7)
1-10%		188 (79)
10-25%		24 (10)
25-100%		10 (4)
Samples		
Ectoparasites		
No	No ectoparasites present in the barn	6 (6)
Psoroptes	Only *Psoroptes* spp. mites present in the barn	31 (29)
Psoroptes + other	*Psoroptes* spp. and other ectoparasites present in the barn	32 (29)
Other	Other ectoparasites present in the barn (*Chorioptes* spp. or lice)	39 (36)
Zn	Zn levels in the blood	
Low		4 (6)
Medium		1 (1)
High		65 (93)
Fe	Fe levels in the blood	
Low		1 (1)
Medium		0 (0)
High		73 (99)
Cu	Cu levels in the blood	
Low		44 (60)
Medium		9 (12)
High		21 (28)
Se	Se levels in the blood	
Low		34 (46)
Medium		12 (16)
High		28 (38)
Barn infrastructure		
Floor type	Floor type in the barn	
Full floor		210 (88)
Slatted floor		19 (8)
Combination		9 (4)
Animal stocking rate		
Tethered	Animals are tethered (no stocking rate)	64 (27)
Box correct	Box with correct stocking rate (≥1m^2/100kg)	130 (55)
Box incorrect	Box with high stocking rate (<1m^2/100kg)	44 (18)
Animal separation		
None	Animals are in 1 box (no separation)	39 (16)
Fence	Animals are separated by a fence (animal contact possible)	162 (68)
Solid wall	Animals are separated by a solid wall (animal contact impossible)	37 (16)
Feeding places	Number of places at the feeding rack	
Insufficient	No place at the feeding rack for some animals	12 (5)
Sufficient	All animals have a place at the feeding rack	226 (95)
Barn climate		
Ventilation system	Ventilation system used in the barn	
Open barn		56 (24)
Closed barn		58 (24)
Slanting intake		59 (25)
Other (e.g. spaceboarding)		65 (27)

Temperature		
Below comfort zone	Barn temperature below comfort zone (<5°C)	17 (7)
In comfort zone	Barn temperature within comfort zone (5-15°C)	157 (66)
Above comfort zone	Barn temperature above comfort zone (>15°C)	64 (27)
Temperature difference	Temperature difference between barn and outdoor environment	
0-1°C		69 (29)
1-2°C		68 (28)
2-4°C		78 (33)
4-8°C		23 (10)
Relative humidity	Relative humidity in the barn	
<70%		149 (63)
70-80%		55 (23)
>80%		34 (14)
Light		
Insufficient	Insufficient light intensity in the barn (<150 lux)	127 (53)
Sufficient	Sufficient light intensity in the barn (>150 lux)	111 (47)
Hygiene		
Straw management	Frequency of replacements of straw bedding	
None (tethered or slatted floor)		62 (26)
<1x/week		2 (1)
Once a week		13 (6)
Minimum 2x/week		76 (32)
Daily		85 (35)
Hygiene score		
Unclean	Hygiene score <2	67 (28)
Clean	Hygiene score ≥2	171 (72)
Feed		
Concentrate		
No	Animals do not get concentrate in the feed	62 (27)
Yes	Animals get concentrate in the feed	43 (17)
Yes (supplemented)	Animals get supplemented concentrate in the feed	133 (56)

*possible dependent variable



CHAPTER 3

Evaluation of two intensive treatment schedules against *Psoroptes ovis* mange in Belgian Blue cattle

Based on: C. Sarre, T. Geurden, J. Vercruysse, N. De Wilde, S. Casaert, E. Claerebout. (2015) Evaluation of two intensive treatment schedules against *Psoroptes ovis* mange in Belgian Blue cattle on nine Flemish farms. Vlaams Diergeneeskundig tijdschrift 84 (6).

3.1 Introduction

Mange is a common parasitic skin disease in Belgian cattle and is potentially caused by 4 mite species: *Demodex bovis*, *Sarcoptes scabiei*, *Chorioptes bovis (C. bovis)* and *Psoroptes ovis (P. ovis)*. *Demodex bovis* lives in the hair follicles as a commensal but can occasionally cause small cutaneous nodules. Infections with *Sarcoptes scabiei* have become rarities in Flemish cattle, while *C. bovis* mainly affects dairy cattle and causes relatively benign symptoms. The most common species in BB beef cattle is *P. ovis* and this mite causes fairly aggressive clinical signs. Moreover, the BB breed seems to be highly susceptible to this infection (Losson *et al.*, 1999). Clinically, an allergic dermatitis is observed, characterized by papules, moist crusts, alopecia and lichenification, especially at the withers, back and tail base. Generalized lesions can occur and often get bacterially infected. Because of an intense pruritus, animals intensely scratch themselves with complications, such as abscesses and hematomas as a consequence (Aiello, 1998; Taylor *et al.*, 2007). Beside an impaired animal welfare, the infestation also leads to production losses due to reduced weight gain, compromised hide quality and increased treatment costs for the farmer (Cole and Guillot, 1987; Lonneux *et al.*, 1998; Rehbein *et al.*, 2003; Stromberg and Guillot, 1989). Psoroptic mange is best treated with an injectable macrocyclic lactone (ML) or topical administration of amitraz, during which all in-contact animals are treated simultaneously and 2 treatments are applied with an interval of 7 to 10 days (Lonneux *et al.*, 1997; O'Brien, 1999; Personne *et al.*, 2006; Taylor *et al.*, 2007; Vercruysse and Rew, 2002). One treatment can be sufficient if the long acting (LA) formulation of moxidectin (10%) is used (Losson *et al.*, 2008). Shearing the animals and removing most of the thick crusts prior to treatment is beneficial for a successful treatment (Plant, 2006).

A 74% farm prevalence of psoroptic mange was demonstrated in a study on Flemish beef farms (Chapter 2). Although many farms are affected, large differences in the severity of the disease are noticeable between individual farms and management factors, such as hygiene (frequency of changing the bedding) and purchase policy may play an important role (Chapter 2). Besides these risk factors, it was also demonstrated that 75% of the questioned farmers implemented an incorrect treatment schedule, during which no diagnosis was made before treatment, an inappropriate product formulation was used, only the clinically affected animals were

treated instead of the whole herd, the animals were only treated once or the interval between 2 treatments was too long (Chapter 2). This could also be the reason of persistent mange problems on certain farms.

The objective of this chapter was to assess whether infestations with *P. ovis* could be controlled on 9 beef farms suffering from chronic mange problems, using one of 2 intensive treatment schedules combined with an optimized farm management.

3.2 Materials and methods

Nine farms with BB animals were followed during one complete housing period (winter 2012-2013, 2013-2014 or 2014-2015) (Table 1). The farms were selected because the farmer or veterinarian stated that mange had been present on the farm for years and that the disease was hard or even impossible to control.

Table 1. Location and number of investigated cattle per farm.

Farm	Province	n cattle*
1	Vlaams-Brabant	50
2	Limburg	40
3	Oost-Vlaanderen	300
4	Oost-Vlaanderen	250
5	West-Vlaanderen	200
6	West-Vlaanderen	350
7	Oost-Vlaanderen	90
8	West-Vlaanderen	15
9	Oost-Vlaanderen	15

*number of investigated cattle

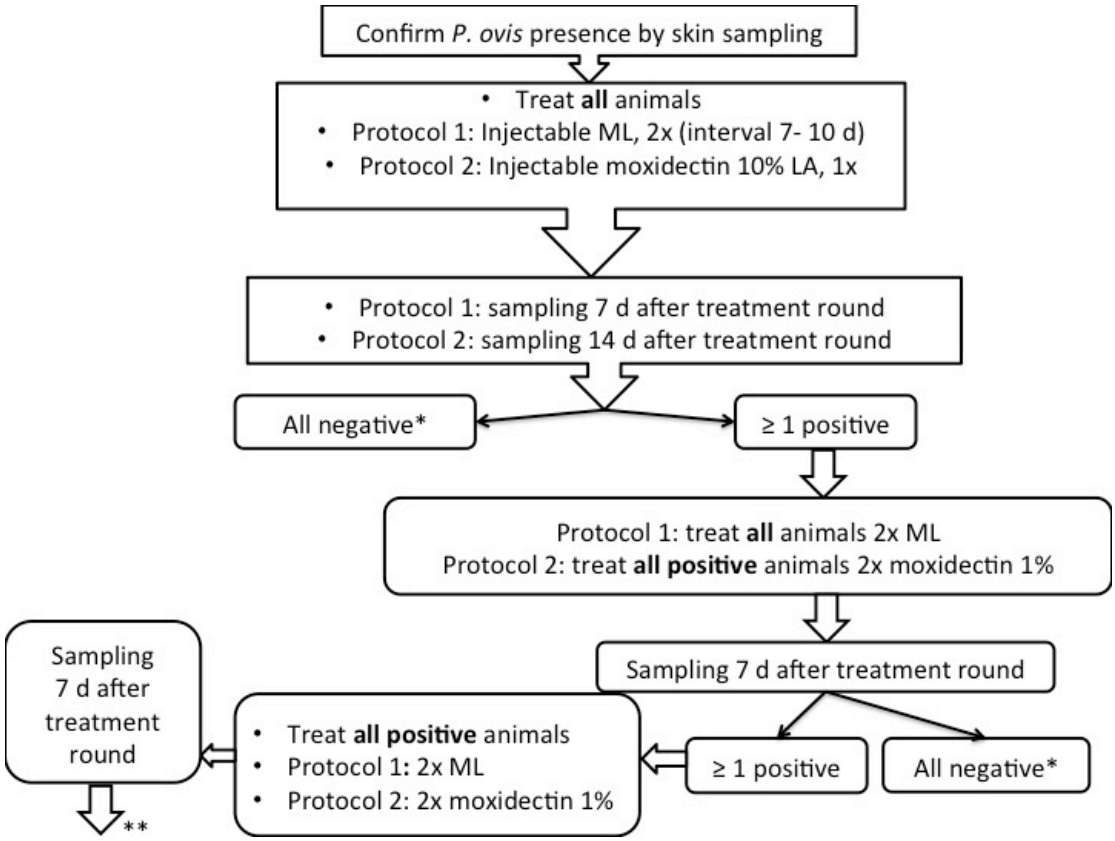
During a first farm visit, skin samples were collected from minimum 5% of the total number of cattle on the farm, spread over all stables, in order to estimate the severity of the problem and to identify the mite species present in each stable (*P. ovis*, *C. bovis* or mixed infection). For each animal, a surface of 3 cm x 2 cm at each clinical lesion was sampled using a scalpel blade. Using a stereomicroscope (25x magnification), these skin samples were screened directly for the presence of live (and mobile) mites, after which 10 living mites from each sample were isolated and clarified in lactophenol to identify the species microscopically. Negative samples were incubated at 37° for 12 hours in 10% KOH (v/v) for indirect examination. After centrifugation (5 minutes at 3000 g), the present mites were enriched by flotation with

a sucrose solution (specific density 1.27) after which they were microscopically identified (100x magnification). Only farms where *P. ovis* or a mixed infection with both *P. ovis* and *C. bovis* was present were followed.

On all farms it was advised to implement specific management measures, such as shearing the animals (at least the back) prior to the first treatment, frequently changing the bedding and treating and isolating purchased cattle for 2 to 3 weeks (Lusat *et al.*, 2009).

Subsequently, one out of 2 treatment protocols was started, in which the animals underwent several treatment rounds (Figure 1). On farms 1 to 7 the first protocol was implemented, starting with the application of a first treatment round on all animals. A treatment round against *P. ovis* mange consists out of 2 treatments of the whole herd with an injectable ML of choice at the prescribed dose with an interval of 7 to 10 days. Ideally, animals should be weighed to calculate the ML dose but in this study the dose for the herd was calculated based on the estimated weight of the heaviest animal (farms 1 – 7). On farms 8 and 9, individual weights were estimated by measuring heart girth (Nantier, 2006). One week after this first treatment round, samples were collected from the visibly affected animals. Only direct examination was performed on these samples as only active infections, characterized by the presence of live mites, needed to be detected after treatment. When a minimum of one sample was positive, another treatment round was administered to all animals. If during the next sampling, one week after treatment, positive animals were still present, only the affected animals were treated in the consecutive treatment rounds due to practical and economic considerations. In the second treatment protocol (farms 8 and 9), the first treatment round consisted of a single injection with the LA formulation of moxidectin (Cydectin 10% LA; Zoetis). Additional treatments were only administered to the affected animals and the regular injectable formulation of moxidectin (Cydectin 1%; Zoetis) was used in each treatment round (2 x with a 7 to 10 days interval). This schedule was applied until all animals were parasitologically negative and/or clinically cured.

Figure 1. Schematic overview of the applied treatment protocols (*follow-up and treatments were stopped; **protocol was continued until all animals were parasitologically negative).



In the first protocol, the treatment efficacy was evaluated by the presence of visible, active clinical lesions (erythema, crusts and/or flaky and wet skin) combined with the presence of live *P. ovis* mites, expressed as the percentage of positive sampled animals. In the second protocol, several complementary criteria were added: a scratching index (ScI) was determined by counting the actions linked to pruritus (licking, scratching, rubbing) during 10 minutes and dividing this total by the number of observed animals. Moreover, a clinical index (CI) was calculated per animal by indicating the infested skin surface on a grid according to the method of Guillot (1981). Finally, for each animal, the number of mites in the skin sample was counted (mite count, MC).

Before turn-out, new skin samples were taken on farms 1 to 7 from minimum 50% of the animals under study. Asymptomatic animals were sampled at the predilection sites for *P. ovis* and *C. bovis* mange. On these farms, another sampling

was performed one year later (at housing) on 5% of the total herd to evaluate the infestation level after being on pasture.

3.3 Results

Farm 2 was the only farm that did not completely implement the advice concerning management, as the animals were not sheared before treatment.

Besides moxidectin LA (farms 8 and 9), the most commonly used drugs were ivermectin and doramectin (farms 1 to 7) (Table 2). On 3 farms (farms 3, 4 and 7) some animals were (additionally) treated twice with amitraz (interval 7 to 10 days) when a mixed infection with *C. bovis* was present or when animals were in the fattening phase and products with a shorter residual activity were required.

Table 2. Number of treatment rounds and used acaricide per farm.

Farm	n cattle*	n Tx**	Acaricide
1	50	3	doramectin
2	40	2	doramectin
3	300	4	ivermectin & amitraz
4	250	3	ivermectin & amitraz
5	200	3	ivermectin & doramectin
6	350	9	ivermectin
7	90	4	ivermectin & amitraz
8	15	4°	moxidectin LA
9	15	3°	moxidectin LA

*number of investigated cattle
**number of treatment rounds to reach 'mange free' status
°first treatment round is a single injection with moxidectin 10% LA and additional treatment rounds are double injections with moxidectin 1% with a 7 to 10 days interval

All farms were declared 'mange free' after one housing period. Farms 1 to 7 were 'mange free' when the whole herd was clinically healthy (Figure 2) and/or when all sampled animals were parasitologically negative during the last farm visit. For the animals on farms 8 and 9 this meant the MC were negative. To reach this 'mange free' status, on average 4 treatment rounds were necessary, with a minimum of 2 and a maximum of 9 treatment rounds (Table 2). The percentage of animals with living mites after each treatment round per farm is listed in Table 3. Table 4 presents the ScI, CI and MC of the positive animals on farm 8 and 9 after treatment. On farms 3, 5

and 7 no control samples could be taken as the last treatments (3, 1 and 1 animal(s) respectively) were given just before the herd went on pasture. Although it was therefore impossible to parasitologically confirm that these farms were 'mange free', the animals were clinically healed. The 7th and 8th injection on farm 6 were administered shortly after the 6th treatment without any interim sampling, because the farmer had noticed clinical signs in an adjacent group shortly after the treatment of the 2 last positive animals and decided to treat all animals, including these last ones.

Figure 2. Example of a BB animal before (left) and after treatment (right).

Table 3. Percentage (%) and number of animals with live mites compared to the total number of sampled animals after each treatment round per farm (n=number of treatment rounds).

n treatment rounds	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7	Farm 8	Farm 9
1*	12% (3/25)	36% (4/11)	22% (8/36)	37% (13/35)	12% (5/42)	44% (12/27)	28% (5/18)	73% (11/15)	53% (8/15)
2	9% (4/46)	0% (0/10)	36% (8/22)	21% (8/38)	4% (1/23)	35% (9/26)	27% (3/11)	40% (6/15)	13% (2/15)
3	0% (0/24)	-	17% (3/18)	0% (0/14)	0% [NS]	15% (2/13)	11% (1/9)	20% (3/15)	0%
4	-	-	0% [NS]	-	-	[NS]	0% [NS]	0%	-
5	-	-	-	-	-	28% (5/18)	-	-	-
6	-	-	-	-	-	35% (8/23)	-	-	-
7	-	-	-	-	-	8% (2/24)	-	-	-
8	-	-	-	-	-	18% (2/11)	-	-	-
9	-	-	-	-	-	0% (0/7)	-	-	-

[NS] no sampling, only clinical evaluation
*first treatment round is single injection with moxidectin 10% LA on farms 8 and 9

Table 4. Arithmetic mean scratch index (ScI), clinical index (CI) and mite counts (MC) of all animals before and after treatment and the number of animals with live mites (n) after treatment on farms 8 and 9.

	Farm 8				Farm 9			
n Tx*	**n**	**ScI**	**CI**	**MC**	**n**	**ScI**	**CI**	**MC**
0	15	3.7	10,3 (3.1–6.6)	953 (6-5590)	15	3.2	10.2 (2.3-20.6)	465 (1-3156)
1	11	3.1	6,2 (1.1-24.9)	102 (0-670)	8	1.5	4,2 (0.3-13.7)	26 (0-250)
2	6	2.4	4,7 (0-21.4)	3 (0-26)	2	0.7	2,1 (0-4.3)	1 (0-10)
3	3	0.7	4,6 (0.9-21.1)	1 (0-4)	0	0.7	3,2 (0-6)	0
4	0	0.5	0,9 (0-3.1)	0	0	0.3	1,1 (0-1.7)	0

*number of treatment rounds (treatment round 1: single injection with moxidectin 10% LA; treatment round 2-4: double injection with moxidectin 1% with a 7 to 10 days interval) In treatment round 1 all animals were treated while in treatment rounds 2-4 only animals with live mites were treated.

At housing, one year after the start of the follow-up, a control farm visit was performed on farms 1 to 7. On all these farms, after being on pasture, new symptoms of mange and live mites were observed on several animals. However, the farmers indicated that the situation was less severe than in previous years: less animals were affected and milder lesions were observed compared to earlier years (smaller lesions and less lesions per animal). All farmers voluntarily applied the first treatment protocol again and stated that the mange problem was under control much faster (2 treatment rounds were usually sufficient).

3.4 Discussion

In this chapter 2 intensive treatment schedules against psoroptic mange were tested on beef farms with chronic mange problems. Although the farm prevalence of mange on Flemish beef farms is very high (74%) according to a previous survey, 'problem farms' such as the farms in the present study are a minority as only 2% of the survey participants indicated that the problem was uncontrollable (Chapter 2).

In only a couple of months, the mange problem on all study farms could be controlled, with all animals being clinically healthy and/or parasitologically negative. In the short term, the application of an intense treatment strategy was therefore successful. It should be stressed that prior to treatment an etiological diagnosis was made, including identification of the mite species, which is essential for an efficient mange control (www.dgz.be/publicatie/fiche-aanpak-schurft).

The number of treatments necessary to reach a 'mange free' situation appeared to be farm dependent and although this is not obvious from this study, larger farms often need more treatments to become completely mite negative. This has been described on sheep farms as well and could be explained by the fact that larger numbers of animals lead to closer contact between individuals and more displacements between stables, both enlarging the risk for dissemination of the infection (Falconi *et al.*, 2002). Moreover, since not all animals were sampled during each farm visit, cattle with small lesions or asymptomatic carriers could easily be missed, especially on larger farms. The results also show that the efficacy of a drug can differ from farm to farm. The presence of farm specific mite strains with a different pathogenicity and/or sensitivity could be a potential cause and this phenomenon has been previously described in sheep and cattle in the US (Roberts and Meleney, 1970). Even though it

was not clear from this study, implementing a suboptimal farm management (nutrition, housing,…) can also be responsible for the persistence of mange on herd level (Chapter 2), as management can influence the immunity or resistance of the animals and as such the response to treatment. Although one ML treatment used to be efficient to control psoroptic mange in the past (Aiello, 1998; Clymer *et al.*, 1997; Lonneux *et al.*, 1997; Pouplard and Detry, 1981), nowadays at least 2 treatments (one treatment round) are necessary to reach clinical improvement (Minihan *et al.*, 2002; Vercruysse and Rew, 2002). The control or eradication of mange often even requires more than one treatment round of 2 ML injections on problem farms with severely affected animals, as in this study. It should be mentioned that the ML dose in this study was determined based on the estimated weight of the animals, thus underdosing is possible in some cases. This cannot only explain therapy failure and the need for more treatments on certain farms, but together with the intensive and often incorrect use of MLs, it can also induce the development of resistant mite strains. Up till now, an *in vitro* test to assess ML resistance in *P. ovis* is not available yet. Despite the clinical and parasitological improvement, additional treatments were also necessary on the farms using the LA formulation of moxidectin. A similar result was obtained in a study from 2012 in the UK, in which a single injection with Cydectin 10% LA was insufficient to control the infection (Mitchell *et al.*, 2012). This is in contrast with a previous study, which demonstrated that in the presence of untreated mange positive animals, cattle remained mange free for a minimum of 77 days after a single injection with the same product (Losson *et al.*, 2008). Nonetheless, an advantage of the LA formulation is that the first treatment round can be replaced by a single injection, which may lower the working load for the farmer. Moreover, the additional treatments on farms 8 and 9 were successful at herd level, despite being only administered to the mite positive animals.

Finally, the question remains how the animals that were negative during winter could become positive during or after being on pasture. The most probable cause is the presence of subclinically infested carriers on the farm. These animals are clinically healthy, but on specific areas they harbour mites that are in a so-called ‘latent phase’, meaning they do not induce clinical signs. In sheep, the infra-orbital and inter-digital fossae, the ears and perineum have been suggested as possible sites where ‘latent’ mites reside (Bates, 2012), but in cattle no such sites have been identified yet. Even when the whole herd would be sampled, these carriers could still

be missed, as 'latent' mites are often not present at the usual predilection sites. Another possible cause is re-infection from the environment. While it was previously assumed that the main infection source for psoroptic mange is direct contact between animals (Bates, 2012; Hourrigan, 1979; Minihan *et al.*, 2002; Losson, 2003), stable hygiene was recently identified as a significant risk factor for psoroptic mange, which implies that re-infection from the environment is possible (Chapter 2). *P. ovis* mites can survive relatively long off the host, but they loose their infectivity after about 2 weeks (Bates, 2012; Pegler and Wall, 2004; Smith *et al.*, 1999). Therefore, it is unlikely that in this study the animals were re-infested on pasture.

It can be concluded that the complete elimination of mange on a BB farm is a difficult task. At short notice, the application of an intensive treatment schedule, combined with an adapted management like shearing prior to treatment is very effective. Because of the possible existence of subclinical carriers or acaricide resistance and the implementation of a suboptimal farm management, the chances that the disease re-emerges in a herd are however high. An early etiological diagnosis is therefore crucial in order to start a correct treatment as soon as possible, to increase the chances for efficient control of the disease.

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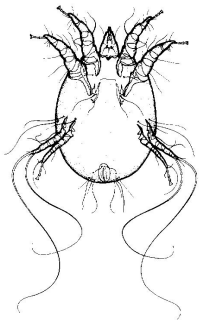


CHAPTER 4

Comparative immune responses against *Psoroptes ovis* in two cattle breeds with different susceptibility to mange

Based on: C. Sarre, A. González-Hernández, S. Van Coppernolle, G. Grit, K. Grauwet, F. Van Meulder, K. Chiers, W. Van den Broeck, P. Geldhof, E. Claerebout. (2015) Comparative immune responses against *Psoroptes ovis* in two cattle breeds with different susceptibility to mange. Veterinary Research, 46:131.

4.1 Introduction

Psoroptes ovis (P. ovis), the causative agent of psoroptic mange, causes severe allergic dermatitis and intense pruritus in sheep and cattle. The disease is highly contagious and causes impaired animal welfare and major economic losses due to performance loss and substantial treatment costs in livestock production all over the world (Kirkwood, 1986; Lonneux *et al.*, 1998; Pouplard *et al.*, 1990). Compared to sheep, *P. ovis* in cattle has a more limited geographical distribution, with a hotspot in Belgium where a farm prevalence of 74% has been observed on Belgian Blue (BB) beef farms (Chapter 2). This cattle breed appears to be highly susceptible to the infection, whilst other beef breeds and dairy cattle, such as Holstein Friesian (HF), seem to be more resistant, although the reason for this difference has not been clarified yet (Losson *et al.*, 1999). Host factors, such as genetic and immunologic variations, could be the cause of different breed susceptibility.

Previous research suggested that immune-depression was not responsible for higher susceptibility of BB, since BB animals developed *P. ovis* specific antibodies after infection, which correlated with the mite population density and the progression of the lesions. Moreover, *in vitro* culture of peripheral blood mononuclear cells (PBMCs) showed an increased reactivity to mitogens (Lonneux *et al.*, 1998; Losson *et al.*, 1988, 1999; Pruett *et al.*, 1986). In general, microscopic examination of the skin from infested ruminants reveals an increased dermal thickness and a superficial, perivascular dermatitis characterised by a rapid influx of lymphocytes (including TCRγδ T-cells in sheep), macrophages, mast cells, neutrophils and eosinophils (Stoeckli *et al.*, 2013; Stromberg and Guillot, 1989; van den Broek and Huntley, 2003; van den Broek *et al.*, 2005). Previous work on sheep scab demonstrated an up-regulation of genes encoding for pro-inflammatory and -allergic mediators (interleukin (IL)-1, IL-8, IL-6) and molecules involved in extravasation of immune cells (Burgess *et al.*, 2010, 2011). Several infectious diseases, including scabies, have been linked to CD4+ helper T (Th)-cell differentiation into specific Th1, Th2, Th17 or Treg lineages (Babu *et al.*, 2010) and the pro-inflammatory response in the skin of *P. ovis* infested sheep evolves towards a Th2 immune response within 24 hours after infection (Burgess *et al.*, 2010, 2011, 2012), a pattern that has been described in *S. scabiei* infested mice and dogs as well (Lalli *et al.*, 2004; Singh *et al.*, 2014). In addition, the epidermal differentiation complex (EDC) genes *filaggrin, involucrin* and

loricrin are significantly down-regulated in infested sheep skin, indicating an impaired skin barrier function (Burgess *et al.*, 2010; Stoeckli *et al.*, 2013) and markers of skin barrier disruption can also be found in circulating PBMCs (Burgess *et al.*, 2012). Furthermore, the systemic immune response parallels the cutaneous Th2 response as locally produced IL-4 and IL-13 stimulate PBMCs to up-regulate IL-4R transcription and CCR3, an eosinophil activator (Burgess *et al.*, 2012). Remarkably, both BB and HF cattle showed an immediate hypersensitivity reaction one hour after intradermal injection of *Psoroptes cuniculi* antigen, but only in BB animals a delayed hypersensitivity reaction after 72 hours could be recorded (Losson *et al.*, 1988, 1999). Inherent differences in skin physiology and body composition could be responsible for this altered skin reaction in BB, as it has been suggested that these physiologic factors can also influence the pharmacokinetics of acaricides (Sallovitz *et al.*, 2002; Vercruysse *et al.*, 2008).

As described for ticks (Piper *et al.*, 2009, 2010), susceptibility of cattle to mite infestations can also vary between individual animals. Increased susceptibility in sheep seems to correlate with higher numbers of eosinophils in the skin, larger lesions (van den Broek and Huntley, 2003) and potentially specific mutations in crucial EDC components (Burgess *et al.*, 2010; Stoeckli *et al.*, 2013). However, the existence of the latter needs further assessment. Humans suffering from severe sarcoptic scabies, better known as crusted or Norwegian scabies, show a predominant IgE-driven Th2 response with high levels of IL-5 and IL-13, in contrast to patients with ordinary scabies, who mainly express the Th1 cytokines IL-2 and IFN-γ (Mounsey *et al.*, 2015; Walton, 2010; Walton and Oprescu, 2013). In mice and rabbits, a skewed Th2/Th1 response favouring the cell-mediated Th1 response is linked with lower antibody titres, also resulting in more resistant individuals (Arlian *et al.*, 1995; Casais *et al.*, 2014; Lalli *et al.*, 2004). Recent investigations demonstrate an important role for Th17 cytokines in humans suffering from chronic and/or allergic inflammatory diseases, such as crusted scabies and psoriasis (Liu *et al.*, 2014). Moreover, in pigs with sarcoptic scabies, the development of severe symptoms is linked to higher levels of cutaneous IL-17, IL-23 but also Th2 cytokines IL-4 and IL-13 (Liu *et al.*, 2014; Mounsey *et al.*, 2015; Walton and Oprescu, 2013).

In conclusion, several potential reasons for differences in susceptibility to mange between breeds and between individual animals have been suggested in various species, with predominant roles for Th2 and Th17 cytokines. Therefore, the

main objective of this chapter was to examine the *in vivo* cutaneous and *in vitro* cellular immune responses during natural *P. ovis* infestation in the highly susceptible BB cattle breed. In addition, these responses were compared with those in more resistant HF cattle.

4.2 Materials and methods

4.2.1 Animals and tissue collection

Ethical approval to conduct this study was obtained from the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (ethical approval number EC 2013/130). In total 28 animals were sampled: 20 BB and 8 HF. All animals were females of approximately one year (BB) or 2 to 3 years (HF) old. The BB cattle were uninfested animals (n=8) or naturally infested animals with severe clinical signs of mange (n=12). The degree of infection was quantified by calculating the percentage of infested body surface (clinical index, CI) for each animal based on the method of Guillot (Guillot, 1981). Heavily infested animals had a CI of ≥10% (range 12-45%) and uninfested animals had a CI of 0%. The HF were also either uninfested (n=4) with 0% CI or naturally infested with *P. ovis* (n=4) with a CI of ≥1% (range 1-8%). Skin scrapings of all infested animals demonstrated a pure *P. ovis* infection. The absence of mites in the uninfested groups was confirmed by indirect examination (centrifugation-flotation after 10% KOH digestion) of skin scrapings.

After confirmation of the presence or absence of *P. ovis* mites, 2 skin biopsies were taken per animal after shaving an area next to the tail base at the transition between healthy and infested skin, using a 4 millimeter (mm) diameter punch biopsy tool (Farla Medical). One biopsy was snap-frozen in liquid nitrogen and stored at -70°C to allow ribonucleic acid (RNA) extraction. The second skin sample was stored in 4% formaldehyde and paraffin-embedded for histology and immunohistochemistry. In addition, blood was drawn from the *vena jugularis* using heparin-coated tubes and PBMCs were isolated using a Lymphoprep density gradient (Axis-Shield). The cells from the interphase were washed 3 times with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen), counted and processed or cultured as described further.

4.2.2 Psoroptes ovis *antigen production*

P. ovis mites of a heavily infested BB animal were collected, washed with DPBS and stored at -70°C. For crude protein extract production, the mites were washed again with ice cold DPBS and crushed in liquid nitrogen in a pestle and mortar. This extract was centrifuged at 16,000 *g* for 30 minutes at 4°C and sterilised over a 0.45 micrometer (µm) filter (Millipore). Using the Bradford method (Sigma-Aldrich), the concentration of the supernatant was determined at 10 milligrams/millilitre (mg/ml) after which the extract was stored at -70°C (Bradford, 1976).

4.2.3 Histology, immunohistochemistry and cell counts

Tissue sections of the paraffin-embedded biopsies were stained with haematoxylin-eosin (HE) for the detection of eosinophils, toluidin blue staining for mast cells and 2 immunohistochemical stainings (CD3 staining for T-cells and CD20 staining for B-cells), based on Dreesen *et al.* (2012). In short, skin tissue sections of 4 µm were mounted on APES-coated slides, blocked with H_2O_2 and stained with polyclonal rabbit anti-human CD3 (Dako) or rabbit anti-human CD20 (Sigma-Aldrich, USA) antibodies. T- and B-cells were visualized by adding peroxidase labelled goat anti-rabbit antibodies (Dako), diaminobenzidin tetrahydrochloride (DAB; Dako) and by performing a counterstaining with haematoxylin. Eosinophils, mast cells, T-cells and B-cells were quantified by taking 2 random pictures per tissue slide at 400x magnification on a *LEICA* light microscope and counting the positive cells on 2 tissue slides per animal. Results were expressed as the number of cells per 10^5 μm^2 tissue surface.

4.2.4 Quantitative real-time PCR

The frozen skin samples that were crushed in liquid nitrogen, homogenized and phase separated in TRIzol (Invitrogen) were used for RNA purification according to Grit *et al.* (2014). In short, RNA was extracted from the aqueous phase using the RNeasy kit (Qiagen). On-column DNase digestion was included by using the RNase-free DNase set (Qiagen) and RNA concentrations were measured with a Nano-Drop 2000 spectrophotometer. In addition, PBMCs were re-stimulated with either DPBS or 5 micrograms (µg)/ml *P. ovis* antigen and cultured for 5 days in a 24-well flat-

bottomed plate (BD Biosciences), after which the harvested cell pellets were washed 3 times with DPBS and stored at -70°C or directly used for on-column RNA purification as described above. Complementary DNA (cDNA) was generated from 100 to 150 nanograms (ng) total RNA using the iScript cDNA synthesis kit (Bio-rad). As the RNA yield from the PBMCs of one uninfested HF was low, 60 ng RNA was used for the cDNA production of the PBMCs from all uninfested HF (n=4). The cDNA of all animals was diluted 1:10 in RNase free water and all qRT-PCR analyses were carried out as described by Dreesen *et al.* (2014) using the SYBR Green Master Mix (Applied Biosystems) on 2 microlitres (µl) of single-stranded cDNA per reaction volume. All reactions were carried out in duplicate. Primer sequences for all genes that were tested are listed in Table 1. Using GeNorm software (geNorm 3.5), 2 breed specific internal control genes were selected from 6 candidate housekeeping genes: glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein P0 (*RLP0*), succinate dehydrogenase complex subunit A (*SDHA*), ribosomal protein 29 (*RPS29*) and histone deacetylase 10 (*HDAC10*). Analysis of the skin samples was performed with housekeeping genes *SDHA* and *RPS29* for BB and *HPRT1* and *SDHA* for HF. Additional genes were tested to evaluate skin related pathology (Table 1). New primer sets for three EDC genes; *filaggrin* (*FIL*), *involucrin* (*IVL*) and *loricrin* (*LOR*) were designed based on the following bovine reference gene sequences from the National Centre for Biotechnology Information (NCBI) database: [*FIL*: XM_010826841, XM_010826842, XM_010826843], [*IVL*: XM_005203832, XM_010802998, XM_005203836] and [*LOR*: NM_001113757, XM_010826802, XM_010802876]. The sequences were aligned in SeqMan Pro (DNASTAR Lasergene) and primers were designed with Primer3web version 4.0.0 (free available online) (Table 1). Mean fold changes in gene transcription levels were obtained by comparing infested with control animals. For the analysis of the PBMCs, housekeeping genes *GAPDH* and *HPRT1* were used for normalization in the BB animals and *RLP0* and *SDHA* for the HF. Relative quantities (Q values) were calculated using the delta Ct method to determine the fold differences in gene transcription levels of antigen re-stimulated cells of each animal compared to DPBS re-stimulated cells.

Table 1. Primer sequences (cattle) and abbreviations of the genes used in the qRT-PCR assays, including GeneBank accession numbers

Housekeeping genes	**Accession number**	**Primer sequence**
GAPDH *(glyceraldehyde-3-phosphate-dehydrogenase)*	*NM_001034034.1*	*F: ACCCAGAAGACTGTGGATGG* *R: CAACAGACACGTTGGGAGTG*
HPRT1 *(hypoxanthine phosphoribosyltransferase 1)*	*NM_001034035.1*	*F: CACTGGGAAGACAATGCAGA* *R: ACACTTCGAGGGGTCCTTTT*
RPS29 *(ribosomal protein 29)*	*BC102702*	*F: GGAGCCATCCGAGAAAATTCG* *R: CAACTTAATGAAGCCGATGTCCTT*
RLP0 *(ribosomal protein P0)*	*NM_001012682.1*	*F: CTTCATTGTGGGAGCAGACA* *R: GGCAACAGTTTCTCCAGAGC*
HDAC10 *(histone deacetylase 10)*	*NM_001075460.1*	*F: CCGATGACGGGAGAAATCTA* *R: CTCAGGAACCCACCAGTTGT*
SDHA *(succinate dehydrogenase complex subunit A)*	*NM_174178.2*	*F: ACATGCAGAAGTCGATGCAG* *R: GGTCTCCACCAGGTCAGTGT*
qRT-PCR PBMC and skin	**Accession number**	**Primer sequence**
IL-2 *(interleukin 2)*	*NM_180997.1*	*F: TCCAAGCAAAAACCTGAACC* *R: CAGCGTTTACTGTTGCATCATC*
IL-4	*NM_173921.2*	*F: GCGGACTTGACAGGAATCTC* *R: TCAGCGTACTTGTGCTCGTC*
IL-5	*NM_173922.1*	*F: TGGTGGCAGAGACCTTGACA* *R: TTCCCATCACCTATCAGCAGAGT*
IL-6	*NM_173923.2*	*F: TCCTTGCTGCTTTCACACTC* *R: CACCCCAGGCAGACTACTTC*
IL-10	*NM_174088.1*	*F: TGTATCCACTTGCCAACCAG* *R: CAGCAGAGACTGGGTCAACA*
IL-13	*NM_174089.1*	*F: GGTGGCCTCACCTCCCCAAG* *R: ATGACACTGCAGTTGGAGATGCTG*
IL-17	*NM_001008412.1*	*F: GGACTCTCCACCGCAATGAG* *R: TGGCCTCCCAGATCACAGA*
IL-23A	*NM_001205688.1*	*F: CCCGTATCCAGTGTGAGGAT* *R: AGTATGGAGGCGTGAAGCTG*
FOXP3 *(forkhead box P3)*	*NM_001045933.1*	*F: GACAGCACCCTTTCGACTGT* *R: CTCCAGAGATTGCACCACCT*
IFN-γ *(interferon γ)*	*NM_174086.1*	*F: TTCTTGAATGGCAGCTCTGA* *R: TTCTCTTCGGCTTTCTGAGG*
NCR1 *(natural cytotoxicity triggering receptor 1)*	*NM_183365.1*	*F: CTGAGAGCGTGGGTGTATCA* *R: CTGAGAGCGTGGGTGTATCA*

TGF-β1 *(transforming growth factor β1)*	*NM_001166068.1*	*F: CTGCTGTGTTCGTCAGCTCT* *R: TCCAGGCTCCAGATGTAAGG*
qRT-PCR skin	**Accession number**	**Primer sequence**
AREG *(amphiregulin)*	*BC141281.1*	*F: TGGTCA* *R: GTCGATCACGGAGGACAGTT*
CCR3 *(chemokine receptor 3)*	*NM_001194960.1*	*F: TGTGTCAACCCCGTGATCTA* *R: AGAGTTCCTGCTCCCCTGTT*
FCERA1 *(Fc IgE receptor alpha 1)*	*NM_001100310.1*	*F: CAGAGGCTGCCCTACATCTC* *R: GTTTAGGCTGTGGGTCCGTA*
FIL *(filaggrin)*	*	*F: GCCCAGTTCTAGACGCTGAC* *R: TCAAGCCAGTGACAGTGAGG*
IVL *(involucrin)*	*	*F: AAGGTCTTGGGCCAGCACTTG* *R: GATGCTGGGTTGTAACTCCCCCCAC*
LOR *(loricrin)*	*	*F: CAGTGGATCCGTCTGCCTGGGA* *R: CATGAGAGCGGTAAGCCCATCGAC*

* Primers designed based on bovine reference gene sequences from NCBI database

4.2.5 Cell proliferation assays

^{3}H-thymidin uptake assay

For the ^{3}H-thymidin uptake assay, 500,000 cells per well were cultured in a 96-well round-bottomed plate (Thermo Scientific) using 200 µl of complete Roswell Park Memorial Institute (RPMI) cell culture medium, which consisted of RPMI-1640 (Invitrogen) supplemented with L-glutamine (Invitrogen), 10% foetal calf serum (Moregate), gentamycin (Invitrogen) and β-mercaptoethanol (Sigma-Aldrich). The cells were either re-stimulated with a negative control (DPBS), 5 µg/ml Concanavalin A (ConA, Sigma-Aldrich) as a positive control or *P. ovis* crude protein antigen at 5 µg/ml, 10 µg/ml, 25 µg/ml or 50 µg/ml. All conditions were performed in triplicate. The cells were pulsed with 1 microcurie (µCi) ^{3}H-thymidin (Perkin Elmer) after 4 days of culture. After 18h of incubation, the cells were harvested and the incorporated radioactivity was measured using a β-scintillation counter (Perkin Elmer). Results are shown as stimulation indices (SI), which are the ratios of the counts per minute of *P. ovis* re-stimulated cells and the counts per minute of the negative control.

PKH staining and flowcytometry

To identify which cell populations from the whole PBMC fraction were proliferating after antigen re-stimulation, isolated PBMCs were labelled with Paul

Karl Horan dye (PKH) using the PKH26 red fluorescent cell linker mini kit (Sigma-Aldrich) according to the manufacturer's protocol. A small fraction of the stained cells was used to determine the PKH starting intensity by flowcytometry analysis. The rest of the labelled cells was cultured at 500,000 cells per well in a 96-well round-bottomed plate (Thermo Scientific) in 200 µl complete RPMI cell culture medium and re-stimulated with DPBS or 5 µg/ml *P. ovis*. All conditions were performed in duplicate.

After 5 days of culture, the cells were harvested and 2 cell stainings were performed in DPBS supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Na-azide (Sigma-Aldrich) by using the following monoclonal primary antibodies: CD3 (mouse IgG1, clone MM1A, VMRD), TCRγδ (mouse IgG2b, clone GB21A, VMRD), CD4 (mouse IgG2a, clone CC8) and CD8 (mouse IgM, clone BAQ111A, VMRD) in the first staining. CD3, CD21 (mouse IgM, clone BAQ15A, VMRD) and Alexa Fluor 488-labeled CD335 (mouse IgG2b, clone AKS6) in the second staining. The CD335 antibody was kindly provided by Prof. A. Storset (School of Veterinary Medicine, Norway). Bound mAb were detected using the following secondary antibodies: rat anti-mouse IgG1-V450 (BD Biosciences), rat anti-mouse IgG1-APC (BD Biosciences), rat anti-mouse IgG2b-FITC (Southern Biotech), goat anti-mouse IgG2a-APC (Invitrogen) and rat anti-mouse IgM-APCCy7 (Biolegend). The PKH intensity for each cell population was determined on a fluorescence-activated cell sorting (FACS)AriaIII (BD Biosciences). Viable cells were gated based on forward and side scatter and lack of propidium iodide (Molecular Probes) uptake. Doublets were eliminated from the analysis by gating on forward scatter-height and forward scatter-area. T-cells were identified as CD3+ cells, T-helper cells as CD3+/CD4+ and cytotoxic T-cells as CD3+/CD8+. In the CD3- population, CD21+ cells were classified as B-cells, CD335+ cells as NK-cells and a third population of bovine CD3-/CD21-/CD335- cells was clearly defined but could not be identified so far.

Data were analysed using FlowJo software (Tree Star) and quantified using ModFit LT software (Verity Software House). Proliferation indices (PI) were calculated for each separate cell population and compared between DPBS and *P. ovis* antigen re-stimulated cells.

4.2.6 Statistical analysis

GraphPad Prism was used to perform all statistical analyses. For the comparison of the stimulation indices and Q values (qRT-PCR on skin biopsies) between infested and uninfested animals, a nonparametric Mann Whitney U test was performed. A one-sided test was used for the stimulation indices and a two-sided test for the Q values. Proliferation indices generated from the PKH staining and Q values of unstimulated vs. re-stimulated cells (qRT-PCR on PBMC) were compared using a nonparametric Wilcoxon signed-rank test: one-sided for the PKH data and two-sided for the Q values. *P*-values ≤ 0.05 were considered statistically significant.

4.3 Results

4.3.1 In vivo *cutaneous immune response*

Results of the eosinophil, T- and B-cell and mast cell counts in the skin of infested and uninfested BB and HF animals are listed in Table 2. The number of eosinophils (46.7 per 10^5 μm^2), T-cells (83.8 per 10^5 μm^2) and B-cells (30.6 per 10^5 μm^2) was significantly higher in the infested BB than in the control animals (6.5 per 10^5 μm^2, 33.8 per 10^5 μm^2 and 5.9 per 10^5 μm^2 respectively), whereas the number of mast cells did not significantly differ. In HF animals, eosinophil, T-cell, B-cell and mast cell counts in the skin appeared to be higher in infested animals compared to the uninfested controls, although cell counts were not significantly different between the groups (Table 2).

Table 2. Histological cell counts in skin biopsies of control animals and *Psoroptes ovis* infested cattle. Panel **A**: Cell counts from Belgian Blue cattle. Panel **B**: Cell counts from Holstein Friesian cattle. Data are presented as mean number of cells per 10^5 μm^2 ± standard error of the mean (SEM). (*P< 0.05)

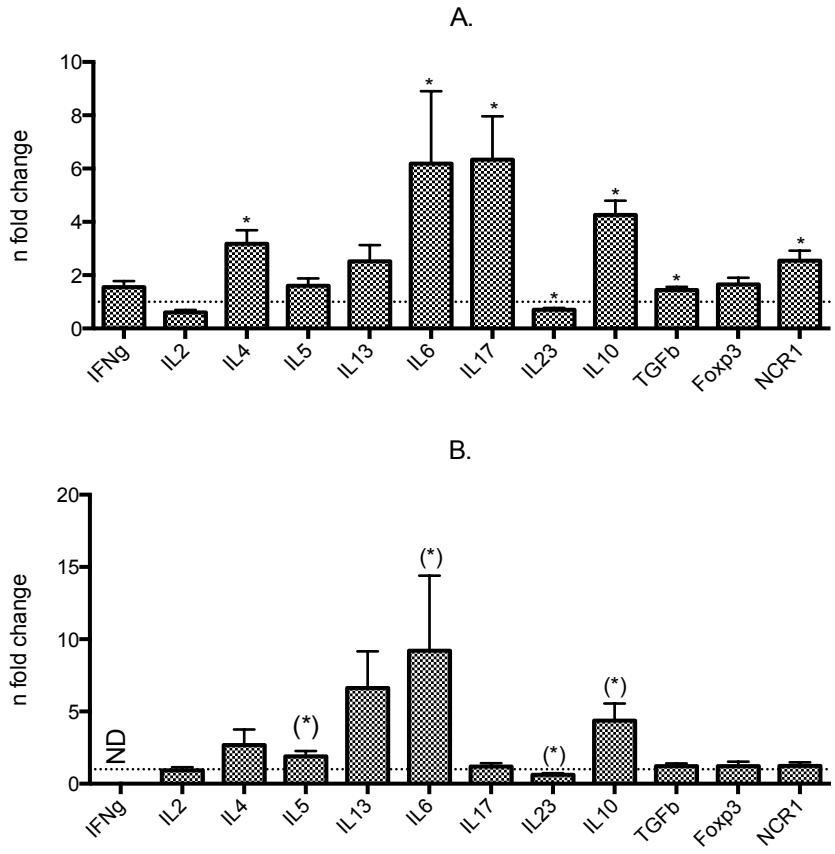
A.	**T-cells (CD3+)**	**B-cells (CD20+)**	**Eosinophils**	**Mast cells**
Control (n=8)	33.8 ± 4	5.9 ± 1.1	6.5 ± 1.3	12.4 ± 1.2
Infested (n=12)	83.8 ± 7.8*	30.6 ± 6.7*	46.7 ± 4.9*	14.3 ± 2.6

B.	**T-cells (CD3+)**	**B-cells (CD20+)**	**Eosinophils**	**Mast cells**
Control (n=4)	34.5 ± 11.7	5.8 ± 0.8	6.4 ± 2.8	12.5 ± 1.2
Infested (n=4)	46.6 ± 5.4	10.6 ± 4	35.1 ± 10	19 ± 4.6

To identify whether specific cytokines characteristic for a Th1, Th2 or Th17 immune response are produced during infection, qRT-PCR was performed on skin biopsies of uninfested and infested BB and HF animals (Figure 1). Only 11 infested BB animals were included as from one animal an insufficient amount of RNA was obtained. A mixed Th2/Th17 cytokine profile was observed in the skin of infested BB with an up-regulation of IL-6 (6.2 fold; P =0.043) and IL-17 (6.3 fold; P =0.006), as well as up-regulated transcription of IL-4 (3.2 fold; P =0.012), IL-5 (1.6 fold; P =0.149), IL-13 (2.5 fold; P =0.107) and IL-10 (4.3 fold; P =0.001). TGF-β and NRC1 were also up-regulated in infested animals (1.4 and 2.5 fold and P-values of 0.006 and 0.003 respectively). Topically transcribed IL-23 was down-regulated (0.7 fold; P =0.019) and a low IFN-γ (1.6 fold; P =0.159) response was observed.

In general, these responses were comparable to those in HF cattle. The cytokine profile in the skin of HF demonstrated a predominant Th2-like response with up-regulated transcription of IL-4 (2.7 fold; P =0.686), IL-5 (1.9 fold; P =0.057), IL-13 (6.6 fold; P =0.124) and IL-10 (4.4 fold; P =0.057). Virtually no change in IL-17 transcription (1.2 fold; P =0.343) was observed, while IL-6 was up-regulated (9.2 fold; P =0.057). No significant changes in IL-23 transcription were observed in the skin (0.6 fold; P =0.057). IFN-γ was up-regulated in 3 out of 4 infested animals, but could not be quantified as the transcription in the uninfested control animals was too low to detect.

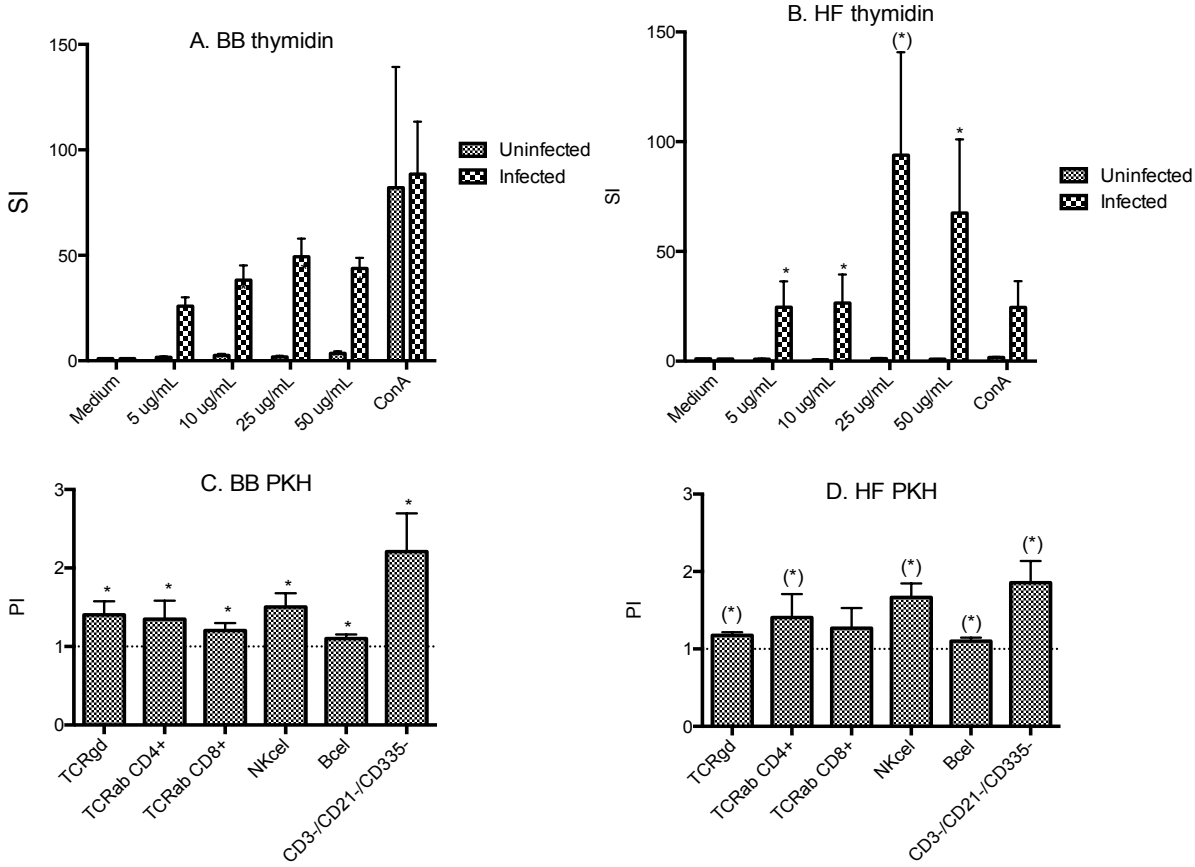
Figure 1. Gene transcription profile in the skin of *Psoroptes ovis* infested Belgian Blue (Panel **A**) and Holstein Friesian cattle (Panel **B**). qRT-PCR data is presented as mean fold changes in infested animals (BB n=11; HF n=4) compared to uninfested controls (BB n=8; HF n=4; dashed horizontal line) with SEM as error bars. (**P*< 0.05, (*)*P*=0.057)



4.3.2 In vitro *immune response after PBMC re-stimulation*

Proliferation of re-stimulated PBMCs was assessed using a ^{3}H-thymidin uptake assay, in which cells from uninfested and infested BB and HF animals were re-stimulated with either DPBS as negative control, ConA as positive control or several concentrations of *P. ovis* antigen (5 µg/ml, 10 µg/ml, 25 µg/ml or 50 µg/ml). Results demonstrated a significant and largely concentration-dependent PBMC proliferation in infested BB and HF compared to the uninfested animals (Figure 2; panels A and B). Using PKH staining on cells of infested BB and HF re-stimulated with 5 µg/ml antigen or DPBS, a significant antigen specific proliferation in all investigated cell populations (αβ T-cells, γδ T-cells, B-cells, NK-cells and CD3-/CD21-/CD335- cells) was observed, with the highest reaction seen in NK- and CD3-/CD21-/CD335- cells (Figure 2; panels C and D).

Figure 2. ^{3}H-thymidin uptake assay and PKH staining of circulating PBMCs from (un)infested Belgian Blue and Holstein Friesian cattle. Panel **A**: mean stimulation index (SI ± SEM) of PBMCs from uninfested (n=8) and infested (n=12) BB cattle re-stimulated with medium, 4 different concentrations of *P. ovis* and ConA. Panel **B**: mean stimulation index (SI ± SEM) of PBMCs from uninfested (n=4) and infested (n=4) HF cattle re-stimulated with medium, 4 different concentrations of *P. ovis* and ConA. Panel **C**: mean proliferation index (PI ± SEM) of PKH labelled PBMCs re-stimulated with 5 µg/ml *P. ovis* antigen compared to unstimulated PBMC (dashed horizontal line) of infested BB (n=12). Panel **D**: mean proliferation index (PI ± SEM) of PKH labelled PBMCs re-stimulated with 5 µg/ml *P. ovis* antigen compared to unstimulated PBMC (dashed horizontal line) of infested HF (n=4). (**P*< 0.05, (*)*P*=0.057)

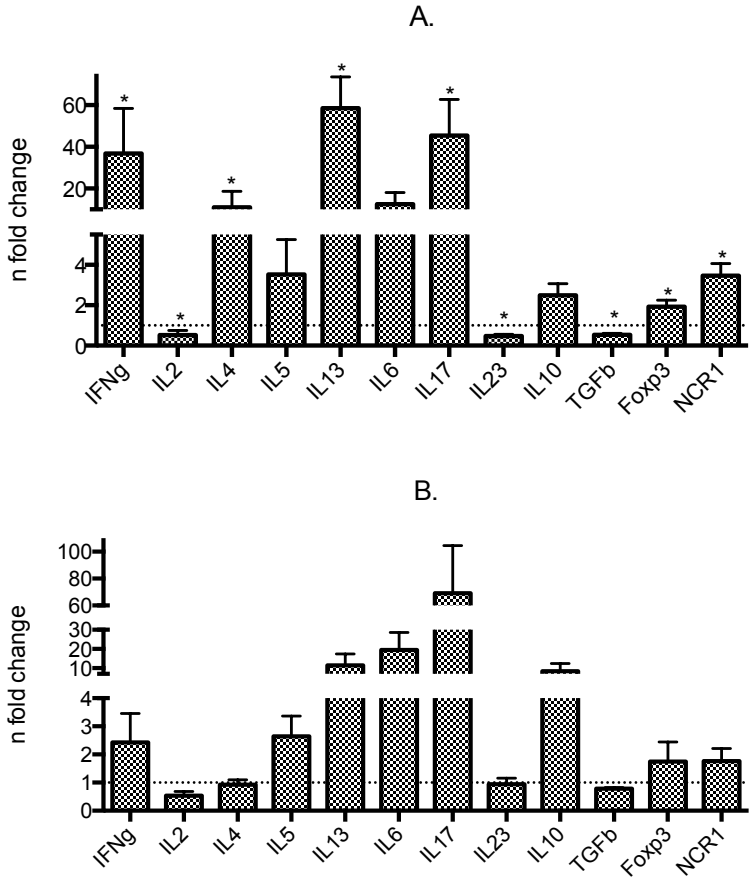


It was subsequently investigated whether circulating re-stimulated PBMCs of infested BB and HF animals after *in vitro* re-stimulation with *P. ovis* antigen produced similar cytokines as in the skin (Figure 3). As the RNA yield of one infested BB animal was insufficient to perform qRT-PCR, data of 11 instead of 12 BB animals is shown in Figure 3; Panel A. In parallel to the cutaneous immune response, re-stimulation of PBMCs in infested BB induced a predominant Th2- and Th17-like response with up-regulated transcription of IL-4 (11 fold; *P* =0.019), IL-13 (58.6 fold; *P* =0.001) and IL17 (45.5 fold; *P* =0.003). In addition, an up-regulation of Foxp3 (1.9

fold; *P* =0.032) and the natural cytotoxicity triggering receptor 1 (NCR1) (3.5 fold; *P* =0.003) transcription was observed, as well as of IFN-γ (36.8 fold; *P* =0.003), IL-5 (3.5 fold; *P* =0.413), IL-10 (2.5 fold; *P* =0.102) and IL-6 (12.5 fold; *P* =0.831). TGF-β, IL-2 and IL-23 were down-regulated (all 0.5 fold with *P*-values of 0.001, 0.005 and 0.001 respectively).

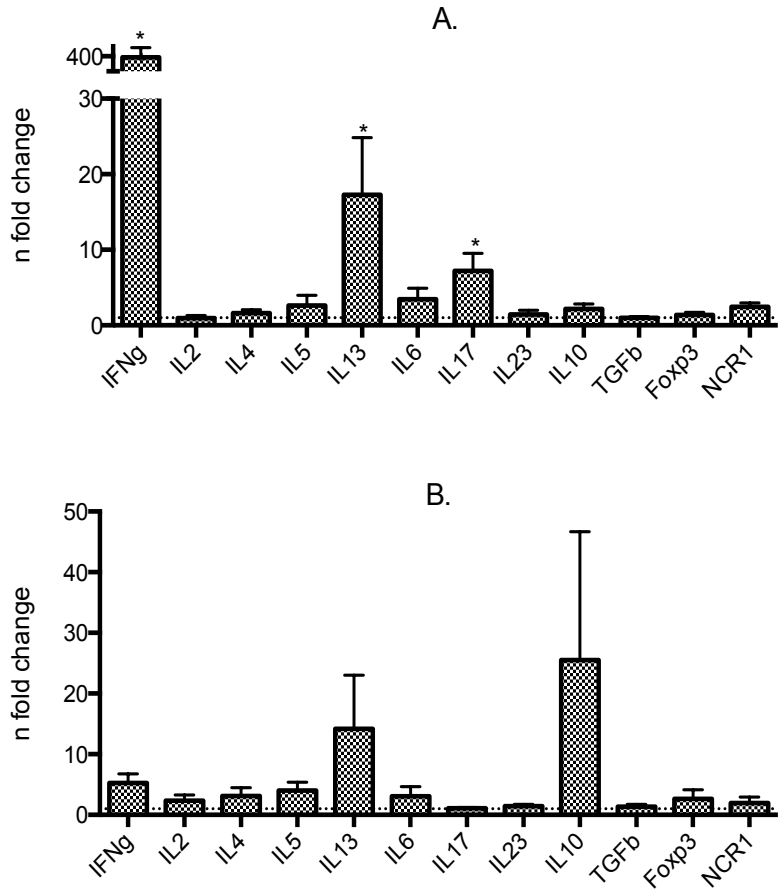
The cytokine profile of circulating re-stimulated PBMCs in infested HF animals (Figure 3; Panel B) revealed a mixed Th2/Th17-like response with 2.6, 11.5 and 19.4 fold changes for IL-5, IL-13 and IL-6 respectively. IL-17 and IL-10 were consistently up-regulated in all samples (average fold changes of 69.1 and 8.5 respectively), systemic IFN-γ was 2.4 fold up-regulated and IL-23, TGF-β and IL-2 were slightly down-regulated, with 0.9, 0.8 and 0.5 fold changes respectively. However, the statistical significance for these data could not be interpreted due to the combination of a low number of animals (n=4) and the use of a two-sided paired statistical test.

Figure 3. qRT-PCR results of circulating PBMCs from infested Belgian Blue and Holstein Friesian cattle. Panel **A**: Gene transcription profile in PBMCs of infested BB (n=11). Panel **B**: Gene transcription profile in PBMCs of infested HF (n=4). qRT-PCR results of PBMC re-stimulated with 5 µg/ml *P. ovis* antigen are presented as mean fold changes in re-stimulated compared to unstimulated cells (dashed horizontal line). (*P< 0.05)



In order to evaluate whether these observations were mainly due to a cellular memory response or whether innate, primary reactions were also responsible, the same procedure was also followed for the uninfested BB (n=8) and HF (n=4) animals with the results listed in Figure 4. A 17.3 fold (P =0.039), 7.2 fold (P =0.031) and 376.8 fold (P =0.039) up-regulation of respectively IL-13, IL-17 and IFN-γ was observed in BB animals (Figure 4; Panel A). Finally, qRT-PCR results from the re-stimulated PBMCs of uninfested HF animals are listed in Figure 4; Panel B and show an up-regulation of IL-13 (14.2 fold) and IL-10 (25.5 fold). In contrast with the PBMCs from uninfested BB cattle, virtually no IL-17 response and a moderate IFN-γ up-regulation (1.9 and 5.3 fold) were observed in uninfested HF. For the results in HF the statistical significance could not be interpreted for the same reasons as mentioned above.

Figure 4. qRT-PCR results of circulating PBMCs from uninfested Belgian Blue and Holstein Friesian cattle. Panel **A**: Gene transcription profile in PBMCs of uninfested BB (n=8). Panel **B**: Gene transcription profile in PBMC of uninfested HF (n=4). qRT-PCR results of PBMCs re-stimulated with 5 µg/ml *P. ovis* antigen are presented as mean fold changes in re-stimulated compared to unstimulated cells (dashed horizontal line). (**P*< 0.05)



4.4 Discussion

In this chapter the cutaneous *in vivo* immune response and *in vitro* cellular immune reaction in BB and HF cattle during natural *P. ovis* infestation were analysed in order to identify a potential cause of the high susceptibility of the BB breed to this parasitic infestation. A significant influx of immune cells in the skin of infested BB was observed, coinciding with a mixed Th2/Th17 cytokine profile. A largely similar cytokine pattern could be elicited when circulating PBMCs from infested BB were re-stimulated with *P. ovis* antigen *in vitro*. Moreover, a significant and largely antigen concentration-dependent PBMC proliferation was observed in infested animals compared to uninfested controls, with proliferation of all cell investigated subpopulations (αβ T-cells, γδ T-cells, B-cells, natural killer (NK)-cells and CD3-/CD21-/CD335- cells). This demonstrates the presence of antigen specific memory T-cells, and it confirms previous assumptions that an impaired cellular immune response

in BB is not the cause of the high susceptibility of this breed (Losson *et al.*, 1999). HF animals are known to display less severe symptoms during *P. ovis* infection in comparison to BB, which is reflected in the lower CI of the animals in this study and the less pronounced influx of immune cells in infested skin. Although the cytokine pattern observed in the skin was similar to that in BB, no cutaneous IL-17 production was observed in infested HF. Furthermore, circulating PBMCs from infested HF also produced a less pronounced Th2-like response. It should however be stressed that a different mite exposure *in vivo* could be responsible for these observations. Furthermore, the infestation stage of the animals was unclear and there was a small age difference between the animals, which could also partly explain the differences between both breeds.

Th2 and Th17 cytokines have been described as being important in parasitic skin diseases. Production of Th2 cytokines is often observed in ectoparasite infections in cattle as it is in dogs, rabbits and mice with scabies (Arlian *et al.*, 1995; Casais *et al.*, 2014; Li *et al.*, 2014; Singh *et al.*, 2014). The cutaneous mixed Th2/Th17 profile that is observed in BB could potentially be associated with more severe symptoms, as described in pigs with aggravating symptoms of *S. scabiei* and in allergic diseases in humans (Mounsey *et al.*, 2015; Li *et al.*, 2014; Liu *et al.*, 2015). Although this specific immune response could merely be a reaction to the presence of severe symptoms linked with high numbers of mites, the reversed hypothesis is also plausible. Indeed, Th2 cytokines produced in the skin will not only stimulate local immune cells to attack the mites, but will also cause collateral damage to the surrounding tissue, leading to the clinical symptoms. Despite the IL-23 down-regulation, the additional pro-inflammatory Th17-like response in BB could intensify these effects leading to a more severe clinical phenotype. As mite counts were not performed in this study, it remains unsure whether this Th2/Th17-like response in infested BB animals is either not protective or is protective but 'uncontrollable'. Little to no signs of a Treg reaction that could dampen this pro-inflammatory response, were noticed in both breeds, as only IL-10, which is also a Th2 cytokine, was up-regulated without transcription of TGF-β. Furthermore, only a diminutive up-regulation of Foxp3, a transcription factor that is generally expressed by Treg T-cells (McNeilly *et al.*, 2010; Murphy, 2012) was noticed. Remarkably, little to no IFN-γ was transcribed in the skin of infested BB, while substantial up-regulation of this pro-inflammatory cytokine was observed in re-stimulated PBMCs from infested and

uninfested BB. The latter indicates that IFN-γ could mainly be released by innate immune cells, such as macrophages, NKT- or NK-cells (Murphy, 2012). Low levels of this cytokine in the skin could be caused by the fact that the adaptive immune response already took over at this point of infection. Production of IFN-γ, a pro-inflammatory cytokine that pushes naïve T-cells towards Th1-cells (Murphy, 2012), has been described in murine spleen and lymph node cells *in vitro* after challenge infection with *S. scabiei* (Lalli *et al.*, 2004). In contrast with the uninfested BB, low IFN-γ and high IL-10 levels were observed in the re-stimulated PBMCs from uninfested HF animals. It remains unclear why IFN-γ and IL-10 transcription in HF does not resemble the pattern observed in BB and it should be further investigated whether this could be part of the cause of different breed susceptibility.

The mixed cytokine profile seen in BB may have an effect on several immune cells downstream. Th2 cytokines are known to promote the immune response to parasites: IL-4 and IL-13 play a role in the induction of allergy as they stimulate B-cell growth and IgE production, whilst IL-5 typically induces eosinophil differentiation and growth (Murphy, 2012). The pro-inflammatory effect of IL-17 causes recruitment of neutrophils and further promotes the attraction of eosinophils in the skin (Iwakura and Ishigame, 2006; Li *et al.*, 2014). Indeed, besides the recruitment of T- and B-cells, cell counts demonstrated a distinct influx of eosinophils in the skin of affected BB and a greater eosinophil skin infiltration has also been documented in sheep breeds susceptible to *P. ovis* and cattle breeds susceptible to tick infestations (Piper *et al.*, 2010; van den Broek and Huntley, 2003). The presence of high levels of IL-17 in the skin has also been linked to delayed-type hypersensitivity in humans (Iwakura and Ishigame, 2006), which could explain the distinctive intradermal skin test response observed by Losson *et al.* (1988 and 1999). In this study, no suppression of the transcription of EDC genes could be observed (results not shown), unlike data available in sheep. As a down-regulation of these genes in sheep skin is seen within 24 hours after infection, it is possible that this was missed in this study (Burgess *et al.*, 2010; Stoeckli *et al.*, 2013).

In summary, BB cattle display a mixed Th2/Th17 immune pathway during natural infection with *P. ovis*. We hypothesize that, in line with results from scabies affected humans and pigs, this might explain part of their susceptibility since no cutaneous Th17 profile could be detected in the more resistant HF breed. Furthermore, in contrast to the results from the HF, high transcription levels of IFN-γ

and low IL-10 transcription in the uninfested and infested BB could indicate a potential role for these cytokines in the innate immune reaction against the mite. These differences in IFN-γ and IL-10 transcription could therefore also be partly responsible for the observed difference in breed susceptibility to mange. Further research is needed to identify potential cell sources and biological functions for these up-regulated cytokines and to fully unravel the basis of this different breed susceptibility to *P. ovis*.

Acknowledgments

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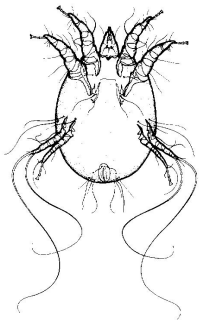

CHAPTER 5

General discussion

Belgian Blue (BB) cattle are highly susceptible to psoroptic mange, leading not only to impaired animal welfare but also important economic losses in the Belgian beef industry. The objective of this thesis was to identify factors that can explain (part of) the susceptibility of BB animals to *Psoroptes ovis*. Mange is a multifactorial problem in which different elements can affect the severity and outcome of the disease. Not only the mite itself is important in this respect but several environmental and host-associated factors can also play a role and were therefore examined in this thesis. This final chapter discusses the results and the potential practical applications from these studies and some suggestions for future research.

5.1 Environmental and parasite factors

5.1.1 Farm management

Psoroptic mange is endemic in Belgium but prevalence data were not available until recently. We confirmed the importance of mange in Belgium by determining a (beef and mixed) herd prevalence of almost 75%, the predominant mite species being *P. ovis* (Chapter 2). Thirteen per cent of the questioned beef farms however indicated to have no problems with mange at all and the severity of the disease varied greatly between farms, which implies that besides the presence of the BB breed, other farm related factors could also be responsible for the variation in severity of mange across farms.

This study demonstrated that poor animal hygiene and purchase of high numbers of new cattle are risk factors for mange. The hygiene score was correlated with the frequency of changing the bedding, which indicates the importance of (re)infection from the environment. Although mange infections mainly spread through direct contact between animals, mites can survive off the host and as such induce a rapid spread of the disease from the environment as well, even though they only stay infective off the host for about 2 weeks (Bates, 1998, 2012; van den Broek and Huntley, 2003). Improving general hygiene measurements, such as frequently cleaning the housing facilities and disinfecting boots and used tools, is therefore advised to avoid severe problems with mange. Purchasing new animals also forms a risk to (re)introduce the infestation as untreated cattle can carry and spread the disease without showing any clinical signs. This finding confirms previous suggestions in cattle and sheep (Berriatua *et al.*, 1999; Carty and Nisbet, 2011; Millar *et al.*, 2011;

Mitchell *et al.*, 2012; Phytian *et al.*, 2013; Pouplard *et al.*, 1990) and emphasizes the need to constrain the purchase of new cattle or at least implement a proper quarantine protocol including treatment and isolation (2 to 3 weeks) after import to avoid infection. The fact that farm management can influence the outcome of mange was no surprise, but we believe that identifying these 2 parameters as actual risk factors is important to improve practical guidelines for mange control in the field. However, additional field tests on a higher number of farms and experimental tests are necessary to confirm these results. A proposal for such an experimental study could be examining the importance of environmental infections by sampling uninfested cattle after introducing them in a pen where infested animals have been kept before. The time between removing the infested and introducing the uninfested animals could be adjusted in order to evaluate the survival and infectivity of *P. ovis* mites in the environment.

5.1.2 Treatment protocols

Although the risk factors discussed above may partially explain the variability between farms, in Chapter 2, 'inadequate treatment protocol' was identified as an additional factor influencing the severity of mange problems. Interestingly, even though a lot of mange treatments are carried out, less than half of the questioned farmers implemented a correct treatment scheme to control the mange problem on their farm. Important aspects of psoroptic mange treatment were identified in Chapter 1, including frequency of treatment, interval between treatments and treating all in-contact animals. Not applying one or more of these elements can lead to treatment failure. Beside not diagnosing the mite species prior to treatment, the most common mistakes are: only treating visibly affected animals, using pour-on formulations of the macrocyclic lactones (MLs) instead of injectable formulations, treating only once or leaving too much time between 2 treatments and not evaluating the efficacy of the treatment(s) (Chapter 2). Therefore, a sensitization campaign in cooperation with Veepeiler (Animal Health Care, Flanders) and Zoetis was launched to stimulate farmers to correctly diagnose and treat mange on their farm (www.dgz.be/publicatie/fiche-aanpak-schurft).

Furthermore, to verify whether the infestation could be eliminated or controlled by implementing an intensive treatment schedule on farms with a history of chronic psoroptic mange problems, 2 treatment protocols were tested (Chapter 3). Although a

correct treatment schedule was applied for the first 4 treatments, due to practical reasons, both protocols were slightly modified by only treating the infested animals during additional treatments. While multiple treatments were often necessary, in all herds the infection could be controlled in a matter of months, which proved both intensive protocols to be effective at short notice. Although it is important to pay attention to as much aspects of an accurate treatment as possible, this study demonstrates that further investigation is needed to evaluate whether treating all in-contact animals at once is necessary as good results were obtained by only re-treating affected cattle. In all cases, the infection reoccurred after summer grazing, most likely due to the presence of subclinical carriers, but the disease seemed to be less severe compared to other years and it was also more responsive to treatment. This result may encourage other farmers to put more effort in the treatment of mange on their farm and it demonstrates that an intensive follow-up leads to satisfying results.

5.1.3 Mite strains

Despite implementing the same treatment and a similar farm management, the number of treatments needed for eradication in the above mentioned study was clearly farm dependent, which suggests that additional factors affect the progress and treatment efficacy of psoroptic mange on farm level. This was also demonstrated by the fact that the only farm that did not follow the management advice (no animal shearing), was able to control mange with the fewest treatments, while on the farm where most treatments were administered, the management was optimal. One of these supplementary influencing factors could be related to the causative pathogen, the *P. ovis* mite. The presence of farm specific mite strains with differing pathogenicity has been described in sheep. High virulent mite strains could be 'non-responsive' even after multiple treatments (Bates, 1997; Roberts and Meleney, 1970). Furthermore, the findings in Chapter 2 demonstrate that acaricides are frequently used and that often-incorrect treatments are carried out in the field, which could lead to the development of acaricide resistance in the mites. *Psoroptes ovis* resistance against ivermectin has already been suspected in cattle (Lekimme *et al.*, 2010), but in contrast to *S. scabiei* (Brimer *et al.*, 1993; Currie *et al.*, 2004; Mounsey *et al.*, 2009; Nong *et al.*, 2014), *in vitro* tests to assess resistance in *P. ovis* are lacking. Therefore, protocols described by Brimer *et al.* (1993) and Nong *et al.* (2014), were adapted for *P. ovis. Psoroptes ovis* mites from 13 Belgian farms were exposed to several concentrations of ivermectin

and compared with susceptible control mites collected from Scottish sheep. Preliminary results showed a farm dependent variety in susceptibility and an increased lethal dose (LD50) on all but one Belgian farms compared to the susceptible control isolate (Maebe, 2015). These results are an indication of the possible existence of ivermectin resistance in Belgium.

5.2 Host related factors

5.2.1 Immune response

Besides farm-related risk factors, host specific factors, such as the immune response, may also partly explain the difference in mange susceptibility that is observed between cattle breeds, e.g. susceptible BB versus more resistant Holstein Friesion (HF) (Bates, 1998; Losson *et al.*, 1999; Pouplard *et al.*, 1990). In Chapter 4, the cutaneous *in vivo* and *in vitro* cellular immune responses in uninfested BB animals were compared with those in infested BB cattle and more importantly these results were compared with those from uninfested and infested HF animals.

Comparison of the **cutaneous immune response** revealed an interesting first difference between both cattle breeds. While in the skin of infested BB a mixed Th2/Th17-like cytokine profile was observed, there was virtually no IL-17 up-regulation in the infested HF. As IL-17 production correlates with the severity of *Sarcoptes* scabies in pigs and allergic diseases in humans (Li *et al.*, 2014; Liu *et al.*, 2014; Mounsey *et al.*, 2015), it is possible that this pro-inflammatory cytokine in BB is responsible for more severe and often generalized clinical symptoms compared to HF. However, IL-17 transcription was observed in peripheral blood mononuclear cells (PBMCs) from HF after *in vitro* stimulation with *P. ovis* antigen. It cannot be excluded that, in contrast to the *in vitro* results, the antigen exposure or number of mites on HF cattle *in vivo* was too low to provoke an IL-17 response in the skin. Unfortunately, no mite counts were performed in this study, so this could not be confirmed. In addition, mange lesions are often secondary infected by bacteria and in general these will elicit a Th17 response in the host (Murphy, 2012). As the BB animals in this study had a higher clinical index compared to the HF, a higher bacterial exposure in the BB could explain the cutaneous Th17 response of infested BB. However, after *in vitro* stimulation of PBMCs with *P. ovis* antigen, a Th17 response was observed in both breeds. This observation was independent of bacterial

exposure, because lipopolysaccharide (LPS) was included in the thymidine uptake assay as a positive control and results showed that this bacterial product had no stimulating effect on the PBMCs (results not shown). Therefore, bacterial contamination is most likely of limited importance in the transcription of Th17 cytokines in the skin of infested BB. Up-regulated cutaneous IL-17 transcription in BB could be responsible for the greater influx of immune cells that was observed in the skin of affected BB compared to HF. The presence of high amounts of IL-17 in the skin of humans has been linked to delayed-type skin hypersensitivity (Iwakura and Ishigame, 2006). A delayed skin test reaction has been observed in infested BB as well, while it was absent in infested HF (Losson *et al.*, 1988, 1999). The observation that only susceptible BB animals elicit a delayed skin reaction after antigen injection is in contrast with observations in *S. scabiei* infested rabbits, in which a delayed reaction, induced by a cell-mediated immune response is often linked with low antibody titres and resistance (Arlian *et al.*, 1994 a and b, 1995).

When the immune responses in **circulating PBMCs** from both BB and HF cattle were evaluated after *in vitro* re-stimulation with *P. ovis* crude antigen, an almost identical memory response was provoked in both breeds. In infested animals a significantly higher PBMC proliferation was observed compared to the controls and all investigated cell populations (αβ T-cells, γδ T-cells, B-cells, natural killer (NK)-cells and CD3-/CD21-/CD335- cells) proliferated. Interestingly, when re-stimulated PBMCs from uninfested BB were compared to those from uninfested HF, 2 cytokines were not transcribed to the same degree: high fold changes of IFN-γ and low transcription levels of IL-10 were observed in PBMCs from uninfested BB animals, while this was reversed in uninfested HF cattle. As this observation was made both in uninfested and infested animals, it was concluded that the pronounced IFN-γ response in BB cattle and the high IL-10 transcription in HF animals most likely are part of the innate immune response and not produced by adaptive Th-cells. The function of this strong, probably innate, IFN-γ up-regulation in BB and IL-10 transcription in HF during infection remains unclear and needs further investigation.

IFN-γ, Th2 and Th17 cytokines are generally thought to all have a pro-inflammatory effect, but host **immune regulatory mechanisms** are thought to step in at some point during infection to dampen the inflammatory response. In this study (Chapter 4), no clear signs of regulatory T (Treg)-cells were noticed in the skin and PBMCs from the 2 cattle breeds and this was reflected in low transcription levels of

TGF-β and Foxp3. In human patients suffering from autoimmune diseases, mutations in Foxp3 have been linked to impaired Treg-cell function and high levels of IL-17 (Passerini *et al.*, 2011a), which corresponds with the pattern seen in cattle in this study. However, it is possible that in our study Treg-cells were missed, because it was uncertain in which stage of infection the naturally infested animals were or because Foxp3 might not be an appropriate cell marker for these cells in cattle. Although Foxp3 is a well-known cell-marker for human and murine Treg-cells, circulating bovine Foxp3+ T-cells have shown to fail induction of a regulatory effect *ex vivo* and Treg-cells in cattle were thought to belong to the TCRγδ T-cell fraction instead, which mainly resides in the skin (Hoek *et al.*, 2009; Van Rhijn *et al.*, 2007).

Various **cell sources** can be responsible for the production of the important cytokines in this study. As IFN-γ transcription differed between BB and HF, the identification of the cell sources of this cytokine could lead to interesting findings. IFN-γ can originate from several innate immune cells, such as macrophages, NKT- and NK-cells (Murphy, 2012). In recent research it has been demonstrated that neutrophils can be a major IFN-γ source in mice, as these innate cells release IFN-γ during degranulation (Sturge *et al.*, 2015).

IL-10 transcription also varied between BB and HF and while IL-10 can be secreted by Th2-cells, it can also originate from mast cells, as has been described in humans suffering from allergic contact dermatitis (Grimbaldeston *et al.*, 2007). The IL-10 up-regulation in the skin of infested BB could be associated with the moderate, although not statistically significant, increase in mast cells. IL-10 can also be released by Treg-cells but as TGF-β and Foxp3 were not up-regulated, the presence of these cells is questionable, as mentioned above. Although not yet identified in cattle, there are specific human Treg-cell subsets (Tr1 cells) that express low levels of Foxp3 and produce IL-10 but little TGF-β (Passerini *et al.*, 2011b; Roncarolo *et al.*, 2001). Finally, keratinocytes and innate immune cells, such as monocytes and macrophages can also release IL-10 (Moore *et al.*, 1993). The fact that this cytokine is known to suppress IFN-γ production by NK-cells (Tripp *et al.*, 1993), could also explain the cytokine pattern seen in uninfested and infested HF.

Investigating the potential cell sources of IL-17 could also clarify the different expression of the cytokine in the skin of BB and HF: IL-17 can possibly originate from Th17 T-cells or several innate immune cells, such as neutrophils, CD8+ T-cells,

NK-like T-cells and TCRγδ T-cells (Stark *et al.*, 2005). TCRγδ T-cell numbers are increased in *P. ovis* infested sheep skin (van den Broek *et al.*, 2005) and in human psoriatic skin (Suzuki *et al.*, 2014) and in the presence of IL-6 these cells can polarize into a specific TCRγδ17 T-cell subset, as described in cattle before (Peckham *et al.*, 2014). These bovine cells are known to produce high levels of IL-6 but not TGF-β and the lack of an IL-23 receptor may explain the non-responsiveness to IL-23 (Peckham *et al.*, 2014). Moreover, in human and mice, these cells are involved in the early pro-inflammatory response with production of IFN-γ, IL-4 and IL-17, stimulation of NK-cells with IFN-γ production and suppression of Treg development (Patil *et al.*, 2015). This cytokine pattern is compatible with the cytokine profile in re-stimulated PBMCs from infested BB.

5.2.2 Genetics

The immunologic differences between BB and HF that were observed in Chapter 4 could be inherent to the breed and therefore have a genetic basis. Even within the BB breed, individual variations in susceptibility to psoroptic mange have been observed and farmers often claim that susceptible dams and sires generate equally susceptible offspring. Similar family ties also seem to exist for more resistant animals, all leading to the assumption that the susceptibility to mange is (partly) heritable.

In this context, our lab contributed to a federal project, called PSOROVIS, of which the ultimate goal was to identify genes that are potentially responsible for mange susceptibility and that could lead to the selection of mange resistant animals on the long run. Before a genome wide association study could be started, a protocol had to be optimized in order to phenotypically distinguish resistant from susceptible animals. A phenotyping formula was generated using a linear equation based on repeated weighed individual mite counts, clinical indices and lesion scores from 670 animals. The individual animal scores were sorted as a continuous variable, with extremely sensitive and resistant animals at the ends and a large 'grey zone' in between (Coussé *et al.*, 2014). After quality control, a single nucleotide polymorphism (SNP) chip analysis was performed on 656 animals and a selection of 150 animals with extreme phenotypes was tested with a 50,000 SNP chip. Preliminary results demonstrated a haplotype at the end of bovine chromosome 11 that seemed to be associated with resistance/susceptibility (Coussé *et al.*, 2015, unpublished results).

This haplotype consisted roughly of 1000 SNPs including the gene for ficolin, a protein that is important for complement activation. The detrimental influence of mange mites on components of the complement system has been demonstrated for *P. ovis* and *S. scabiei* (Bergstrom *et al.*, 2009; Burgess *et al.*, 2010; Fischer *et al.*, 2009; Holt *et al.*, 2003; Maruo *et al.*, 1997) and a genetically fixed divergent complement activation could therefore have an influence on the mange susceptibility of an individual animal or an entire breed.

5.3 Conclusion and future perspectives

This thesis identified several factors that might contribute to the susceptibility of BB cattle to psoroptic mange. At first, the importance of mange on Flemish beef farms was confirmed by determining a (beef and mixed) herd prevalence of 74%. Besides demonstrating the negative influence of an incorrect treatment, this study also identified poor hygiene and purchase of high numbers of animals as risk factors for the occurrence of mange problems on farm level. In order to confirm the importance of these specific management factors and to potentially identify other risk factors, the execution of a similar study on more farms or experimental trials could be beneficial. One of those additional risk factors could be the feeding of (supplemented) concentrate, as animals in the fattening period often seem to be free of mange or appear to display less severe symptoms. As inconclusive results were obtained for this parameter in our risk analysis, research on a higher number of farms could lead to more significant results.

Despite a similar farm management and treatment protocol, differences in acaricide efficacy could be observed between individual farms. This observation suggests the potential existence of more virulent or resistant mite strains. The development of an *in vitro* test to assess ivermectin resistance in *P. ovis* led to promising preliminary results (Maebe, 2015) and suggests that on some farms ivermectin resistant mites could be present. However, this test should be further optimized and since a threshold for acaricide resistance is currently unavailable, future research is necessary to determine such a cut-off value. In addition, the test should be validated on more farms and the *in vitro* results should be correlated with *in vivo* efficacy results. When acaricide resistance is suspected in specific mite populations, the next step will be to identify the molecular base of this resistance. As

MLs function through interaction with the GluCl and potentially the GABA-gated chloride channels of the parasite (Vercruysse and Rew, 2002), it would be interesting to compare the molecular structure and function of these features in resistant and susceptible mites. Besides GluCl-gated chloride channels, adenosinetriphosphate (ATP) binding cassette (ABC) transporters, such as P-glycoproteins, could be of interest as well, since these have been frequently mentioned as being important in ML resistance in helminths (Demeler *et al.*, 2013; Ouellette, 2001; Prichard and Roulet, 2007). As far as amitraz concerns, another frequently used acaricide in the treatment of mange, potential changes in the target of this drug, the octopamine receptor, are worth looking into (Chen *et al.*, 2007). However, in this respect it should be mentioned that current research is limited to the expansion of a *P. ovis* cDNA library based on expressed sequence tag (EST) analysis (Burgess *et al.*, 2011; Kenyon *et al.*, 2003; Lee *et al.*, 1999) and the lack of a full genomic *P. ovis* DNA sequence might hamper future research on the molecular base of acaricide resistance.

Besides environmental and mite-related factors, host-inherent aspects may also contribute to the mange susceptibility or resistance of a breed or individual animals. This study demonstrated a lack of IL-17 up-regulation in the skin of infested HF compared to BB, and PBMCs from BB but not HF seemed to elicit a substantial, presumably innate, IFN-γ response after re-stimulation with *P. ovis* antigen. In order to further investigate these breed dependent differences and to fill some of the gaps of this study, some future research suggestions are proposed in the following paragraph.

A pitfall in this study was the mite exposure in the naturally infested animals from both breeds most likely not being identical and as this could influence the *in vivo* results from the skin biopsies, mite counts should be included in future studies when naturally infested animals are used. Another option is to perform experimental infections, during which equal mite loads are used in both breeds. This would allow a longitudinal follow-up of the immune responses from the moment the mites are administered on the skin, until a few weeks after experimental infection. During this period, skin biopsies and PBMCs could be processed as in our previous research. Alternatively, a microarray study (Burgess *et al.*, 2010) or RNA-sequencing could be used to study the dynamics of the immune responses in BB and HF in more detail. At this stage, RNA-sequencing would be preferred as whole genome sequences of cattle and sheep (Daetwyler *et al.*, 2014; Jiang *et al.*, 2014) are available and the costs for this type of research are reduced. In order to further standardize the antigen load on

the skin, instead of mites, intradermal or topical application of *P. ovis* antigen could be considered. Such an intradermal skin test (IDT) was already optimized for BB animals (results not shown). Based on the test used by Losson *et al.* (1988, 1999) an optimal antigen concentration was determined, i.e. a concentration that did not elicit a reaction in uninfested animals. This optimized IDT was performed on 100 BB animals in an attempt to classify animals as resistant or susceptible based on their IDT reaction. Preliminary results of this test were inconclusive but the test could be used for other purposes in future research. After intradermal injection or topical application of the antigen, the immune response of both BB and HF animals could be evaluated by taking skin biopsies at regular time points and processing them as described above.

Another drawback was that it was uncertain in which stage of infection the naturally infested animals were at the time of sampling and that only a 'snapshot' of the cutaneous immune response was evaluated. This could also be avoided by using experimental infections. The memory immune response mounted by *in vitro* re-stimulated PBMCs was also only assessed at one time point, i.e. after 5 days in culture. In order to investigate the immune responses more closely over time and to see whether the cytokine profiles change over time after *in vitro* re-stimulation, proliferation and cytokine production of PBMCs could be evaluated at several evaluation points, for example after 24 hours, 3 days, 5 days and 7 days in culture.

Thirdly, thorough investigation of the upstream mechanisms that lead to the immune responses that were identified during this study and of downstream mechanisms that may execute the effector response and/or regulate the inflammation, is needed in order to explain the differences between the 2 investigated cattle breeds and to completely understand the pathogenesis of psoroptic mange. Upstream investigation could focus on the interaction of antigen presenting cells (APC) with T-cells, which gives an indication on which mite antigens are processed by APC and how the innate immune response models the adaptive immunity in a certain direction. The cellular sources of the observed cytokines in this study and the downstream (in)direct effects of these cytokines on the mites and potentially the host itself are also interesting pathways to investigate. More information on the IFN-γ cell sources could be obtained by including neutrophil counts in future research, as these cells have been mentioned as being important IFN-γ producers (Sturge *et al.*, 2015).

It would also be interesting to examine whether the differences that were observed in the immunologic study have a genetic basis. The breed specific up-regulated

cutaneous Th17 response or innate IFN-γ up-regulation could be heritable within the BB and therefore it would be beneficial to link the results from the PSOROVIS project to these findings. However, further research is necessary to confirm and refine the results of the genome wide association study, to potentially identify important genes and to see whether susceptibility to psoroptic mange is linked to structural proteins, such as ficolin or other proteins that might influence the immune response towards a Th17 profile or IFN-γ production. Even if the immune responses during infection could be described in detail over time, further research would still be necessary to demonstrate whether these counteract or contribute to the development of clinical signs. A corticosteroid test could be used to see whether the immune response is responsible for the lesions and whether dampening or interrupting this effector response by local and/or systemically applied corticosteroids would have an effect on lesion development.

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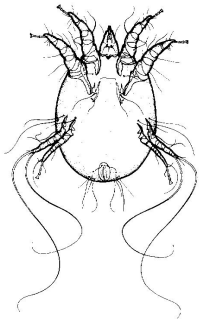


SUMMARY

Summary

Psoroptes ovis mites cause psoroptic mange, a skin disease that affects sheep and cattle worldwide. Clinically an allergic dermatitis with yellow wet crusts, pruritic erythematous skin and alopecia can be observed and while symptoms typically occur at the withers, back and tail base, they can also spread covering the entire body (generalized form). The infection does not only lead to impaired animal welfare, but also to substantial economic losses, especially in beef cattle. The Belgian Blue (BB) beef breed is highly susceptible to psoroptic mange but the reason why these animals suffer more from the infection compared to dairy cattle remains unclear. Besides genetic, immunologic or physiologic differences inherent to the breed, environmental factors, such as farm management, may also be responsible for the susceptibility of these animals.

In **Chapter 1**, the available literature on *P. ovis* is reviewed with the focus on pathogenesis, epidemiology, diagnosis, treatment and the host immune reactions during infection. This review demonstrates that, although psoroptic mange is a multifactorial disease, little information is available about the environmental parameters that may influence the outcome of the disease and that may explain the susceptibility differences between BB farms. Therefore, the first objective of this thesis was to identify specific farm management factors, including treatment protocol, that could act as risk factors for mange and that could explain the difference in severity of the disease between BB farms. Because mange problems can differ considerably, even within farms between individual animals, host related factors are also thought to contribute to the susceptibility of the BB breed in general. The second objective was therefore to study the immune responses during infection in BB animals and to compare these with those in the less susceptible Holstein Friesian (HF) cattle.

Because mange problems vary significantly between farms, a cross-sectional questionnaire survey and subsequent farm visits were performed in **Chapter 2** in order to identify potential risk factors for *P. ovis* infections on BB farms in Flanders (Belgium). The questionnaire was sent to 1,800 beef farms to evaluate the presence and severity of psoroptic mange in the herd and to assess farm management practices, including antiparasitic treatments. Subsequently, about 10% of the farms with a completed questionnaire were visited to validate the questionnaire and to retrieve supplementary information on additional management parameters, such as barn infrastructure and climate. Associations between parasitism and putative risk factors were assessed by logistic regression. Out of 1,800

contacted farms, 680 (38%) completed questionnaires were received. Data were collected from 238 barns during 66 farm visits. The questionnaire results demonstrated a high farm prevalence of mange (74%; 95% CI (70.7 – 77.3)) and half of the farmers declared that the problem was difficult to control. Nevertheless, in only 14% of the barns a high scratching index was recorded and in most of the sampled animals (80%) the affected body surface was less than 10%. This indicates that despite the high prevalence and the difficulty to control the infection, clinical signs were often quite moderate. Logistic regression analyses of the questionnaire and the farm visit data suggested that heavily infested farms treat more intensively against mange. On most farms, mange occurred the whole year round and more problems with mange were found on farms where a higher number of animals were purchased per year. In addition, the disease was more prevalent when the animals had a lower hygiene score. This score was strongly correlated with environmental hygiene, indicating that transmission of mites from the environment to the animals should not be underestimated. Conflicting results were obtained on the effect of supplementing minerals on the occurrence of mange. In this study, temperature, light intensity and relative humidity in the barns, ventilation systems, barn infrastructure, animal stocking rate and blood mineral levels were not indicated as risk factors for mange. In conclusion, maintaining a good animal hygiene and if possible, avoiding introduction of cattle may help in the control of psoroptic mange in Belgian Blue cattle.

In the field, treatment failure is often allocated to acaricide resistance, but from the survey in Chapter 2, it became clear that most farmers use an incorrect treatment schedule in the control of the infection by treating affected animals only, using pour-on formulations instead of injectables or leaving more than 10 days between 2 treatments. The objective of **Chapter 3** was therefore to evaluate whether an intensive treatment schedule could control the infection on farm level. Two intensive treatment schedules were evaluated on BB farms with a persistent mange problem. On farms 1 to 7, all animals were treated twice (7 to 10 days interval) with an injectable macrocyclic lactone (ML), while on the 2 remaining farms the initial treatment consisted of one injection with the long acting (LA) formulation of moxidectin (10%). Skin scrapings were taken one week after treatment and when living mites were found on at least one animal, all animals (farms 1-7) or only positive animals (farms 8 and 9) were treated consecutively with an injectable ML. On all farms, treated animals were clinically healthy and *P. ovis* free at the end of winter, after 2 to 9 treatment rounds (2 injections with 7-10 days interval or one LA injection). Although mange reappeared on the

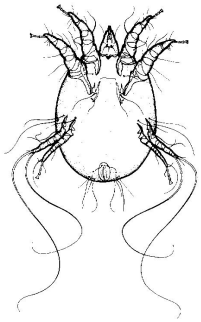
first 7 farms after the subsequent grazing season, the disease was less severe and easier to control.

Besides the farm related factors described in Chapter 2 and 3, host related immune responses could also be a cause of the natural predisposition of the BB beef breed to psoroptic mange. In **Chapter 4**, the *in vivo* cutaneous immune response and *in vitro* cellular immune response after antigen re-stimulation were examined in naturally infested BB. Cytokine transcription in the skin and in circulating re-stimulated PBMCs demonstrated a mixed pro-inflammatory Th2/Th17 profile, with transcription of IL-4, IL-13, IL-6 and IL-17. Strong IL-17 up-regulation in the skin of BB was associated with an influx of eosinophils and other immune cells, potentially leading towards more severe symptoms. Virtually no changes in cutaneous IFN-γ transcription were detected, while there was substantial IFN-γ up-regulation in re-stimulated PBMCs from infested and uninfested animals, potentially indicating a role of this pro-inflammatory cytokine in the innate immune response. In HF cattle, a largely similar immunologic response was observed. Differences between HF and BB were the lack of cutaneous IL-17 response in infested HF, the low transcription levels of IFN-γ and the clear up-regulation of IL-10 transcription in re-stimulated PBMCs from both infested and uninfested animals. Further research is needed to identify potential cell sources and biological functions for these cytokines and to fully unravel the basis of this different breed susceptibility to *P. ovis*.

Finally, in **Chapter 5**, all results, some drawbacks of this thesis and potential future perspectives are debated. Besides confirming the importance of psoroptic mange in Belgium, this thesis could identify several factors (animal hygiene and import of new animals) that might partly explain the difference in mange problems between individual BB farms. As the number of examined farms was limited, similar research on additional farms and experimental studies are however necessary to confirm that management elements may influence the outcome of the disease on farm level. Another important factor was treatment protocol. Therapy failure is often observed under field conditions and although the presence of acaricide resistant mites should not be refuted, this thesis demonstrated that farmers often implement an incorrect treatment schedule, potentially explaining the lack of treatment efficacy. Therefore, a sensitization campaign in cooperation with Animal Health Care Flanders was launched, which focussed on correctly diagnosing and treating psoroptic mange (www.dgz.be/publicatie/fiche-aanpak-schurft). Additionally, the evaluation of 2 intensive

treatment schemes on 9 farms with mange problems showed that psoroptic mange could be controlled, although a high number of treatments were sometimes required. The results from this study can encourage other farmers to apply a correct treatment protocol as the favourable results may compensate for the intensive treatment protocol. However, despite similar management and treatment protocols, mange problems can still vary significantly between farms. This indicates that besides environmental factors, mite and host related aspects might also play a role in the high susceptibility to mange. Due to the frequent but often incorrect use of MLs, ivermectin resistance is already suspected in Belgian psoroptic mites. This was supported by preliminary results from an *in vitro* test demonstrating a decreased and highly variable susceptibility between mites originating from 13 Belgian farms compared to a susceptible control isolate. In the future, this *in vitro* test should be optimized, used on more farms and linked with *in vivo* efficacy data. In the long-term, the molecular mechanisms of ML resistance in the mites should be addressed, in order to develop molecular detection tests. Concerning host related influencing factors, this thesis confirmed that BB had a different immunologic response during *P. ovis* infection when compared to HF cattle. Future research should focus on confirming these results, revealing the upstream mechanisms and cell sources of important cytokines and their downstream effects and functions. In addition, some drawbacks of this work should be tackled in future studies: mite exposure should be standardized and quantified by performing mite counts and a longitudinal follow-up of the immune response after infection should be established by performing experimental infections and/or by including more evaluation points for the *in vitro* PBMC work. Finally, the genetic basis of the differing immune response should be identified, in view of genetic selection of mange-resistant animals.

In conclusion, this thesis has revealed several potential causes of the high susceptibility of BB cattle towards psoroptic mange and practical guidelines could be generated to eliminate farm related risk factors. However, future research to specify and identify the BB specific immunity towards *P. ovis* is needed in order to construct practical tools, such as immunotherapy or vaccination, for the control of psoroptic mange.



SAMENVATTING

Samenvatting

De ectoparasiet *Psoroptes ovis* veroorzaakt schurft, een huidziekte die wereldwijd zowel schapen als runderen kan aantasten. Klinisch wordt een allergische dermatitis met gele natte korsten, jeukende erythemateuze huid en alopecie opgemerkt. Hoewel symptomen typisch ter hoogte van de schoft, rug en staartbasis voorkomen, kunnen deze spreiden tot een gegeneraliseerde vorm waarbij het volledige lichaam aangetast is. De infectie heeft niet alleen een verminderd dierenwelzijn tot gevolg, maar ook belangrijke economische verliezen, voornamelijk in de vleesvee-industrie. Het Belgisch Witblauwe (BB) runderras is overgevoelig aan *Psoroptes* schurft, maar de reden waarom deze runderen erger lijden onder de infectie in vergelijking met melkvee blijft onduidelijk. De hypersensitiviteit van BB dieren kan veroorzaakt worden door genetische, immunologische en fysiologische verschillen die eigen zijn aan het ras, maar ook omgevingsfactoren zoals bedrijfsmanagement kunnen mede verantwoordelijk zijn voor verschillen tussen BB bedrijven onderling.

In **hoofdstuk 1** werd de beschikbare literatuur rond *P. ovis* samengevat. Onderzoek rond verschillende aspecten zoals epidemiologie, behandeling en immuniteitsopbouw toont aan dat *Psoroptes* schurft een multifactoriële ziekte is, maar dat er weinig gekend is over specifieke omgevingsfactoren die potentieel de overgevoeligheid van het BB ras mede kunnen verklaren. Daarom was de eerste doelstelling van deze thesis bedrijfs-afhankelijke managementfactoren, waaronder het behandelingsprotocol, te identificeren als mogelijke risicofactoren voor schurft. Deze factoren zouden het verschil in schurftproblematiek tussen individuele BB bedrijven kunnen verklaren, maar het probleem blijkt eveneens sterk te verschillen tussen individuele dieren binnen eenzelfde bedrijf. Dit impliceert dat specifieke gastheerfactoren vermoedelijk ook bijdragen aan de overgevoeligheid van het BB ras. De tweede doelstelling van deze thesis was daarom de immuunresponsen tijdens infectie bij BB dieren te evalueren en deze te vergelijken met die in meer resistente Holstein Friesian (HF) dieren.

Doordat schurftproblemen sterk kunnen verschillen tussen individuele bedrijven onderling, werden in **hoofdstuk 2** een enquête en bijhorende bedrijfsbezoeken uitgevoerd met als doel risicofactoren voor *P. ovis* infecties te identificeren op Vlaamse BB bedrijven. De enquête werd naar 1800 vleesveebedrijven gestuurd en bevatte vragen over de aanwezigheid en ernst van *Psoroptes* schurft in de kudde en over bedrijfsspecifieke managementfactoren, zoals antiparasitaire behandelingen. Vervolgens werd ongeveer 10% van de bedrijven met een

volledig ingevulde enquête bezocht ter validatie van de vragenlijst en om extra informatie te bekomen over bijkomende management-parameters zoals stalinfrastructuur en -klimaat. Associaties tussen parasitisme en mogelijke risicofactoren werden onderzocht door middel van logistische regressie. Van de 1800 bedrijven stuurden er 680 (38%) een complete vragenlijst terug en tijdens 66 bedrijfsbezoeken werd data verzameld van 238 stallen. Uit de enquêteresultaten kwam een hoge bedrijfsprevalentie van schurft naar voor (74%; 95% CI (70.7 – 77.3) en de helft van de veehouders gaf aan dat het probleem moeilijk tot niet te controleren was. Nochtans werd slechts in 14% van de stallen een hoge 'scratch index' gemeten en bij de meeste bemonsterde dieren (80%) bedroeg het aangetaste lichaamsoppervlak minder dan 10%. Dit toont aan dat klinische symptomen vaak vrij mild zijn ondanks de hoge prevalentie en de moeilijk te controleren aard van de infectie. De logistische regressie analyse suggereerde dat er op zwaar geïnfecteerde bedrijven ook meer behandeld wordt tegen schurft. Op de meeste bedrijven kwam de infectie het ganse jaar door voor en de grootste problemen werden vastgesteld op bedrijven waar jaarlijks grote aantallen nieuwe dieren aangekocht werden. Bovendien was de ziekte vaker aanwezig wanneer de dieren een lage hygiënescore hadden. Deze score was sterk gecorreleerd met de omgevingshygiëne, wat aantoont dat de overdracht van mijten vanuit de omgeving naar de dieren niet onderschat mag worden. Tegenstrijdige resultaten werden bekomen omtrent het effect van mineralen supplementatie en tevens konden temperatuur, lichtintensiteit, relatieve vochtigheid in de stallen, ventilatie-systemen, stalinfrastructuur, bezettingsdichtheid en mineralen-bloedwaarden niet aangeduid worden als risicofactoren voor schurft. Er kon geconcludeerd worden dat het behouden van een goede hygiëne en het zo min mogelijk aankopen van nieuwe dieren kunnen helpen bij de controle van *Psoroptes* schurft op BB vleesveebedrijven.

In de praktijk wordt therapiefalen vaak aan de aanwezigheid van acariciden-resistente mijten geweten. Uit hoofdstuk 2 bleek echter dat op de meeste bedrijven een incorrect behandelingsschema gebruikt wordt om de infectie te controleren, waarbij enkel de klinisch aangetaste dieren behandeld worden, pour-on formulaties in plaats van injecteerbare producten gebruikt worden of meer dan 10 dagen tussen 2 behandelingen gelaten wordt. Het doel van **hoofdstuk 3** was dan ook te evalueren of een intensieve behandeling toch in staat was om een infectie op bedrijfsniveau volledig te controleren. Daarom werden 2 intensieve behandelingsschema's toegepast op 9 Vlaamse vleesveebedrijven met een persisterend schurftprobleem. Op bedrijven 1-7 werden alle dieren 2 maal behandeld (interval 7 à 10

dagen) met een injecteerbaar macrocyclisch lactone (ML). Op de 2 laatste bedrijven werd een éénmalige injectie toegediend met de 'long acting' (LA) formulatie van moxidectine (10%). Eén week na behandelen werden huidafkrabsels genomen en bij aanwezigheid van levende mijten bij ten minste 1 dier werden alle dieren (bedrijven 1-7) of alle positieve dieren (bedrijven 8 en 9) verder behandeld met een injecteerbaar ML. Op alle bedrijven waren de dieren klinisch genezen en schijnbaar vrij van mijten na 2 tot 9 behandelingsrondes (2 injecties met 7-10 dagen interval of 1 LA injectie). Hoewel er na het daaropvolgende weideseizoen op de eerste 7 bedrijven opnieuw tekenen van schurft te zien waren, was dit in mindere mate en gemakkelijker te controleren.

Naast de omgevingsfactoren die aan bod kwamen in hoofdstuk 2 en 3, kan een gastheer-specifieke immuunrespons ook een mogelijke oorzaak zijn van de natuurlijke predispositie van BB runderen voor *Psoroptes* schurft. Daarom werden in **hoofdstuk 4** de cutane immuunrespons (*in vivo*) en de cellulaire immuunrespons (na antigen herstimulatie *in vitro*) onderzocht in natuurlijk geïnfesteerde BB dieren. De cytokine productie in de huid en de circulerende geherstimuleerde PBMCs vertoonden een gemengd pro-inflammatoire Th2/Th17 profiel, met verhoogde transcriptie van IL-4, IL-13, IL-6 en IL-17. De sterke IL-17 transcriptie in de huid van geïnfecteerde BB was geassocieerd met een influx van eosinofielen en andere immuuncellen, wat mogelijks de ergere symptomen bij BB kan verklaren. Hoewel er zo goed als geen IFN-γ transcriptie gedetecteerd kon worden in de huid, werd dit cytokine duidelijk wel afgeschreven door geherstimuleerde PBMCs van zowel geïnfecteerde als niet-geïnfecteerde dieren. Dit suggereert een mogelijke rol voor dit pro-inflammatoire cytokine als onderdeel van de aangeboren of 'innate' immuunrespons. Bij HF runderen werd een grotendeels identieke immuunrespons opgemerkt. De grootste verschillen tussen HF en BB dieren waren het gebrek aan een cutane IL-17 respons bij geïnfecteerde HF, de lage transcriptiewaarden van IFN-γ en de verhoogde transcriptie van IL-10 in de geherstimuleerde PBMCs van zowel geïnfecteerde als niet-geïnfecteerde HF dieren. Verder onderzoek naar de cellulaire oorsprong en biologische functies van deze cytokines is noodzakelijk om de basis van de verschillende ras-afhankelijke gevoeligheid voor *P. ovis* bloot te leggen.

Tot slot worden in **hoofdstuk 5** alle resultaten, enkele nadelen van de gebruikte methodologie en potentiële toekomstperspectieven aangehaald. Naast het bevestigen van het belang van *Psoroptes* schurft in België (74% bedrijfsprevalentie), kon deze thesis verschillende factoren ('slechte hygiëne' en 'aankoop van hoge aantallen nieuwe dieren')

identificeren die mogelijks een deel van de gevarieerde gevoeligheid ten opzichte van *P. ovis* tussen BB bedrijven kunnen verklaren. Aangezien het aantal onderzochte bedrijven beperkt was, zijn gelijkaardig onderzoek op bijkomende bedrijven en experimentele studies nodig om te bevestigen dat deze managementfactoren effectief een invloed hebben op de problematiek op bedrijfsniveau. Een bijkomende belangrijke parameter was het behandelingsprotocol tegen schurftmijten. Therapiefalen komt onder praktijkomstandigheden vaak voor en ook al kan de aanwezigheid van acariciden-resistente mijten niet uitgesloten worden, toch kon in deze thesis aangetoond worden dat veehouders vaak een incorrect behandelingsschema toepassen op hun bedrijf, wat de lage behandelingsefficiëntie mogelijks kan verklaren. Daarom werd in samenwerking met Diergezondheidszorg Vlaanderen een sensibiliseringscampagne op punt gesteld waarbij de focus op een correcte diagnose en behandeling van *Psoroptes* schurft ligt (www.dgz.be/publicatie/fiche-aanpak-schurft). Bovendien toonde de evaluatie van 2 intensieve behandelingsprotocols op 9 probleembedrijven aan dat *Psoroptes* schurft wel degelijk kon gecontroleerd worden, hoewel vaak veel behandelingen nodig waren. Aangezien een intensieve behandeling tot goede resultaten leidde, kunnen de resultaten van deze studie een stimulans zijn voor andere veehouders om een correct behandelingsschema toe te passen in de strijd tegen schurft. Ondanks een gelijkaardig management, behandelingsschema incluis, kan een schurftprobleem toch sterk verschillen tussen BB bedrijven onderling. Dit impliceert dat naast omgevingsfactoren, ook mijt- en gastheer-specifieke aspecten een rol kunnen spelen in de gevoeligheid voor schurft. Door het frequent, maar vaak incorrect gebruik van MLs, werd ivermectine resistentie reeds vermoed bij Belgische *Psoroptes* mijten. Dit werd bevestigd door preliminaire resultaten van een *in vitro* test, waarbij de gevoeligheid van mijten afkomstig van 13 Belgische bedrijven sterk varieerde en verlaagd was in vergelijking met een gevoelig controle-isolaat. Toekomstig onderzoek dient te focussen op de optimalisatie van deze *in vitro* test, op de validatie van de test op een groter aantal bedrijven, op het verband tussen *in vitro* resultaten en *in vivo* efficaciteitsdata en op het identificeren van het moleculair mechanisme van resistentie in de mijten. Wat betreft gastheer gerelateerde factoren, kon deze thesis bevestigen dat BB dieren een verschillende immunologische reactie vertoonden in vergelijking met HF vee. Terwijl bij de geïnfecteerde BB een gemengde Th2/Th17 cutane respons opgemerkt werd, was IL-17 transcriptie in de huid van geïnfesteerde HF afwezig. Verder kon een sterke en waarschijnlijk aangeboren IFN-γ respons gedetecteerd worden in de geherstimuleerde PBMCs van geïnfecteerde en niet-geïnfecteerde BB dieren terwijl dit niet te zien was bij HF. Om deze resultaten te bevestigen, de onderliggende mechanismen te identificeren en de downstream effecten en functies van deze cytokines aan

te tonen, is echter meer onderzoek nodig. Sommige nadelen van de gebruikte methodologie zouden hierbij ook aangepakt kunnen worden: de mijten-blootstelling zou gestandaardiseerd en gekwantificeerd moeten worden en een longitudinale opvolging van de immuunrespons na infectie zou gerealiseerd moeten worden door experimentele infecties uit te voeren en/of door meer evaluatie tijdstippen bij het *in vitro* PBMC werk in te lassen. Bovendien dient in toekomstig onderzoek aandacht besteed te worden aan de mogelijke genetische basis van de verschillende immuunrespons die geobserveerd werd, met het oog op genetische selectie van schurft-resistente dieren.

Deze thesis heeft verschillende potentiële oorzaken van de overgevoeligheid van BB rundvee ten opzichte van *Psoroptes* schurft geïdentificeerd en praktische richtlijnen gegenereerd om bedrijfsafhankelijke factoren te beperken. Verder onderzoek is echter nodig om de specifieke immuniteit die BB dieren tegenover *P. ovis* opbouwen verder te specifiëren en zo finaal praktische tools, zoals immunotherapie of vaccinatie, te kunnen voorzien voor de controle van *Psoroptes* schurft.